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Fungi in Pentachlorophenol Adsorption and Degradation: Novel Bioremediation and Biotechnological Tools

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“Non abbiate paura di andare controcorrente!

Siate coraggiosi!”

Papa Francesco

Abstract

Pentachlorophenol (PCP) has been used worldwide as a wood preservative and pesticide. PCP toxicity and extensive use has placed it among the worst environmental pollutants, and therefore its microbiological degradation to develop bioremediation techniques has been intensively studied.

The current study, part of a wider bioremediation project, is a long-term evaluation of the remediation ability of naturally selected microorganisms versus PCP in laboratory-scale experiments. The main aims of this thesis were: i) to define PCP sensitivity, adsorption and degradation of *Byssosclamyces nivea* (Westling 1909), *Scopulariopsis brumptii* (Samson and Klopotek 1972) and *Anthracoerythron discolor* (Mont. Singer 1951) in microbiological culture media; ii) to test *B. nivea* and *S. brumptii* as antagonists against two Oomycetes: *Phytophthora cinnamomi* and *Phytophthora cambivora*; iii) to evaluate the response of an agricultural soil to PCP, with or without compost (biostimulation) and *B. nivea* and *S. brumptii* (bioaugmentation), in terms of effects of the contaminants on the main chemical, biochemical and biological soil properties.

B. nivea and *S. brumptii* showed a good PCP tolerance (12.5 and 25 mg PCP L⁻¹) although hyphal size, biomass, patulin and spore production decreased for increasing concentrations of PCP. It was shown that these two fungi can completely deplete 12.5 and 25 mg PCP L⁻¹ in a submerged culture after 28 days of incubation at 28 °C. Electrolyte Leakage Assays showed that the fungi have a good tolerance at 25 mg PCP L⁻¹. *B. nivea* and *S. brumptii* were able to inhibit the growth of *P. cinnamomi* and *P. cambivora* on solid media and in liquid culture. Volatile organic compounds (VOCs) did not produce growth reduction of oomycetes strains.

The PCP that was adsorbed by *A. discolor* pellets was >80% compared to pH values of 5 and 5.5, which were the two concentrations being analyzed. PCP adsorption significantly decreased in a medium of pH > 6.0. The Infrared Spectroscopy (FTIR)

results showed that amides, alkanes, carboxylates, carboxyl and hydroxyl groups may possibly be important to the PCP adsorption for pellets of *A. discolor*.

In microcosm soil experiment, the addition of PCP severely depressed some of the tested biochemical properties (i.e. microbial biomass, soil respiration, dehydrogenase activity and fluorescein diacetate hydrolysis) suggesting an inhibitory effect on microbial activity. The compost had a buffer effect against the PCP, limiting the decrease of soil biochemical activity vs. of the control. After 28 day of incubations the compost and the fungal strains reduced of 95% the extractable PCP. The natural fresh soil showed a good capacity of reduction of extractable PCP (88%). The main soil processes (i.e. microbial degradation, biostimulation by compost and sorption to organic matter) were likely occurred in the contaminated soil when was added PCP and are involved in PCP depletion.

Our results indicate that *B. nivea* and *S. brumptii* have an interesting potential for bioremediation and biocontrol strategy. *A. discolor* may be used as a natural biosorbent for liquid solutions which are contaminated by PCP.

Key words: PCP, Biological control, Soil biochemistry, Biostimulation, Bioaugmentation.

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Contents

Chapter I

1. General introduction and research objectives	1
1.1. Pentachlorophenol: chemical identification	1
1.2. Use and production of PCP and derivatives	1
1.3. Toxicity of PCP	5
1.4. Environmental contamination by PCP	6
1.5. Biological methods	7
1.6. Microbial degradation of PCP	9
1.7. Research objectives	10
1.8. References	11

Chapter II

2. A comprehensive overview of bacteria and fungi used for pentachlorophenol biodegradation	18
2.1. Abstract	18
2.2. Abbreviations	19
2.3. Introduction	19
2.4. PCP degrading by bacteria	22
2.4.1. The genus <i>Acinetobacter</i>	23
2.4.2. The genus <i>Bacillus</i>	24

2.4.3. The genus <i>Desulfitobacterium</i>	26
2.4.4. The genus <i>Flavobacterium</i>	27
2.4.5. The genus <i>Kokuria</i>	28
2.4.5. The genus <i>Mycobacterium</i>	29
2.4.6. The genus <i>Pseudomonas</i>	30
2.4.7. The genus <i>Serratia</i>	35
2.4.8. The genus <i>Sphingomonas</i>	38
2.4.9. Other genera	42
2.4.10. Bacteria general discussion	45
2.5. PCP degrading by fungi	52
2.5.1. The genus <i>Anthracoephyllum</i>	52
2.5.2. The genus <i>Mucor</i>	54
2.5.3. The genus <i>Penicillium</i>	55
2.5.4. The genus <i>Phanerochaete</i>	57
2.5.5. The genus <i>Pleurotus</i>	59
2.5.6. The genus <i>Rhizopus</i>	61
2.5.7. The genus <i>Trametes</i>	62
2.5.8. The genus <i>Trichoderma</i>	65
2.5.9. Other genera	65
2.5.10. Fungi general discussion	69
2.6. Concluding remarks	75

2.7. Future perspectives	78
2.8. Refernces	80

Chapter III

3. Effect of pentachlorophenol concentration and pH levels on the adsorption capacity of <i>Anthracophyllum discolor</i> pellets	98
3.1. Abstract	98
3.2. Introduction	99
3.3. Materials and Methods	101
3.3.1. Fungal strain and cultivation conditions	101
3.3.2. Preparation of the fungal pellets	101
3.3.3. Surface area measurement	101
3.3.4. Potentiometric titration of <i>A. discolor</i> cell wall	102
3.3.5. PCP adsorption	102
3.3.6. Fourier transforms Infrared spectroscopy	103
3.3.7. Assessing <i>A. discolor</i> growth and Remazol Brilliant Blue R decolorization	103
3.3.8. Statistic analysis	104
3.4. Results and Discussion	104
3.4.1. Morphological characteristics of <i>A. discolor</i> pellets	104
3.4.2. Potentiometric titration	105
3.4.3. Effect of PCP concentration and pH levels on the adsorption capacity of <i>A. discolor</i>	107

3.4.4. FTIR analysis	111
3.4.5. Assessing <i>A. discolor</i> growth and Remazol Brilliant Blue decolourization	114
3.5. Conclusions	115
3.6. References	115

Chapter IV

4. Assessing the effectiveness of <i>Byssochlamys nivea</i> and <i>Scopulariopsis brumptii</i> in pentachlorophenol removal and biological control of plant pathogens	122
4.1. Abstract	122
4.2. Introduction	122
4.3. Materials and Methods	124
4.3.1. Isolation of fungal strains	124
4.3.2. DNA extraction, PCR amplification and analysis	125
4.3.3. Chemicals	126
4.3.4. Sensitivity test to PCP in plate culture	126
4.3.5. Sensitivity test to PCP and PCP removal in submerged culture	126
4.3.6. Utilization of PCP as sole carbon source	127
4.3.7. Influence of PCP on production of patulin in submerged culture	127
4.3.8. Electrolyte Leakage Assay (ELA)	128
4.3.9. Antagonistic assays against <i>Phytophthora</i> spp. in vitro	129
4.3.10. Statistical analysis	130
4.4. Results	130

4.4.1. Fungal strains identification	130
4.4.2. Sensitivity test to PCP	130
4.4.3. PCP removal capacity	133
4.4.4. Electrolyte Leakage Assay	133
4.4.5. Antagonistic assays against <i>Phytophthora</i> spp. in vitro	134
4.5. Discussion	135
4.5.1. <i>B. nivea</i> and <i>S. brumptii</i> in PCP sensitivity	135
4.5.2. <i>B. nivea</i> and <i>S. brumptii</i> in PCP degradation	137
4.5.3. Biological control of <i>P. cambivora</i> and <i>P. cinnamomi</i>	139
4.6. Conclusions	141
4.7. References	141

Chapter V

5. Bioaugmentation and biostimulation of an agricultural microcosm soil contaminated by pentachlorophenol	149
5.1. Abstract	149
5.2. Introduction	150
5.3. Materials and Methods	152
5.3.1. Fungal strain and cultivation conditions	152
5.3.2. Chemicals	152
5.3.3. Physical and chemical properties of soil and compost	152

5.3.4. Soil biochemical analysis	153
5.3.5. PCP depletion in microcosm soil experiment	153
5.3.6. PCP extraction and quantification	154
5.3.7. Statistical analysis	155
5.4. Results	156
5.4.1. Physical and chemical properties of soil	156
5.4.2. Biochemical properties	157
5.4.3. PCP depletion	162
5.5. Discussion	164
5.6. Conclusions	167
5.7. Refernces	168
Chapter VI	
6. Concluding remarks	174

List of figures

- Fig. 1 Potentiometric titration curves of live pellets of *A. discolor* with three KCl electrolyte solutions at 25 ± 1 °C. 106
- Fig. 2 Potentiometric titration curves of live pellets of *A. discolor* in a KCl 0.1M electrolyte solution at 25 ± 1 °C with different concentrations of PCP. 107
- Fig. 3 Effect of pH levels on the adsorption capacity of *A. discolor* pellets (mean \pm standard deviation) at 25 ± 1 °C for 24 hours of (a) 5 mg PCP L⁻¹ (b) 10 mg PCP L⁻¹. Different small letters refer to significant differences ($P < 0.05$) among PCP sorption values. 109
- Fig. 4 Spectrum of FTIR analysis of *A. discolor* pellets. Solid black line indicates FTIR of only *A. discolor* biomass while, broken grey line indicates FTIR of *A. discolor* biomass after sorption of 10 mg PCP L⁻¹ at 25 ± 1 °C for 24 hours. Capital letter marked significative troughs of functional groups. 113
- Fig. 5 Toxic effects of 25 mg PCP L⁻¹ on 6 plugs of *B. nivea* and *S. brumptii* after 24 h of incubation using Electrolyte Leakage Assay (ELA). Results are expressed as mean \pm standard deviation. Different small letters refer to significant differences ($P < 0.05$) among PCP sensitivity of: *B. nivea* incubated in distilled water (white bar); *S. brumptii* incubated in distilled water (light grey bar); *B. nivea* incubated at 25 mg PCP L⁻¹ (black bar); *S. brumptii* incubated at 25 mg PCP L⁻¹ (dark grey bar). 134
- Fig. 6 Toxic effects of *B. nivea* and *S. brumptii* culture filtrates on 10 tomato stem pieces after 24 h of incubation using Electrolyte Leakage Assay (ELA). Results are expressed as mean \pm standard deviation. Different small letters refer to significant differences ($P < 0.05$) among toxicity on tomato stem incubated in: only PDB (control) (white bar); *B. nivea* culture filtrates (black bar); *S. brumptii* culture filtrates (grey bar). 135
- Fig. 7 Soil respiration of: a) control (without PCP);b) PCP contaminated sample (25 mg Kg⁻¹). 157
- Fig.8 Microbial biomass of: a) control (without PCP); b) PCP contaminated sample (25 mg Kg⁻¹). Different small letters refer to significant differences ($P <$ 158

0.05) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

Fig. 9 Fluorescein diacetate hydrolysis of: a) control (without PCP); b) PCP 160
contaminated sample (25 mg Kg^{-1}). Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

Fig. 10 Dehydrogenase activity of: a) control (without PCP); b) PCP 161
contaminated sample (25 mg Kg^{-1}). Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bars represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

Fig. 11 Extracted PCP at different incubation times. Different small letters refer 163
to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 7 days (grey light bar); 14 days (black bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

List of tables

Table 1. Chemical identity of PCP	2
Table 2. Physical and Chemical Properties of PCP	4
Table 3. Degradation of PCP by bacteria	49
Table 4. Degradation of PCP by fungi	72
Table 5. Sensitivity test to PCP by fresh fungal mycelium at different PCP concentrations. Different small letters refer to significant differences ($P < 0.05$) between PCP sensitivity of <i>B. nivea</i> and <i>S. brumptii</i> at the same PCP. Different capital letters refer to significant differences ($P < 0.05$) among PCP sensitivity of <i>B. nivea</i> and <i>S. brumptii</i> at different PCP.	131
Table 6. Adsorption and degradation of PCP (12.5 and 25 mg L ⁻¹) in 100 ml of PDB by fresh fungal mycelium at different PCP concentrations. Different small letters refer to significant differences ($P < 0.05$) between removal PCP values of <i>B. nivea</i> and <i>S. brumptii</i> at the same PCP concentration. Different capital letters refer to significant differences ($P < 0.05$) among removal PCP values of <i>B. nivea</i> and <i>S. brumptii</i> at different PCP concentration	131
Table 7. Antagonistic assays of <i>B. nivea</i> and <i>S. brumptii</i> against <i>Phytophthora</i> spp. in vitro. Different small letters refer to significant differences ($P < 0.05$) between growth values of <i>P. cambivora</i> or <i>P. cinnamomi</i> versus the same conditions of antagonism. Different capital letters refer to significant differences ($P < 0.05$) among growth values of <i>P. cambivora</i> or <i>P. cinnamomi</i> versus different conditions of antagonism.	132
Table 8. Agricultural microcosm soil experimental design. The symbols correspond: ● 30 plug (5 mm of diameter) of active mycelia from 7 day old cultures on PDA medium; □ 15 plug (5 mm of diameter) of active mycelia from 7 day old cultures on PDA medium for each fungus; * 7.14 g compost Kg ⁻¹ soil; ▲ 25 mg PCP Kg ⁻¹ soil.	155
Table 9. Main physical-chemical properties of the soil.	156



Fomes fomentarius

1. General introduction and research objectives

1.1. Pentachlorophenol: chemical identification

Over the last fifty years the environmental pollutants have been a significant problem worldwide because represent a hazard for the health and survival of all organisms (Kimani 2007). Many organic pollutants are chemical substances of anthropogenic origin that are hard to eliminate in the environment and resistant to biodegradation processes found in naturally occurring microorganisms (Jones and de Voogt 1999).

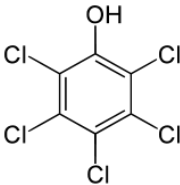
Pentachlorophenol (PCP) is an artificial semivolatile organochlorine compound that is not generated naturally in the environment (Rappe et al. 1979). It is a white crystalline solid with phenolic odour, soluble in organic solvents but with low water solubility (14 mg L^{-1}) and with a density greater than water ($1.98 \text{ g cm}^3 \text{ }^{-1}$) (Shiu et al. 1994). It melts at $190 \text{ }^{\circ}\text{C}$ and boils at $300.6 \text{ }^{\circ}\text{C}$ (Table 1 and 2). It is easily subjected to photodegradation above all by ultraviolet radiation (Suegara et. al 2005).

1.2. Use and production of PCP and derivatives

Biocide low cost, the PCP has been added to adhesives, paints, food cans, storage containers, resins, lubricants, photographic solutions, (EPA 1984; Carrizo et al. 2008), detergent, supplement in medical soap (Abramovitch and Capracotta 2003), herbicides, algaecides, molluscicides, bactericides and fungicides (Routt Reigart and Roberts 1999; Hong et al. 2010; Huang et al. 2011). A greater use was also made in leather, pulp, paper and wood industry for preservation of the timber and bleaching of paper or tissues (Gadd 2001; Singh 2006; Rubilar et al. 2008; Ruder and Yiin 2011). This substance, abundantly used in agricultural and industrial purposes, since its introduction in 1936 by Dow Chemical Company and Monsanto Chemical Company (Cedar, 1984). Still today is used and produced in some countries (Pointing 2001), it is

marketed under many trade names as Santophen, Penchlorol, Chlorophen, Pentacon, Penwar, Sinituho and Penta (Morgan 1989). It can be found essentially in two forms: PCP and as the sodium salt of PCP. In 1985, the world wide production of PCP was 100.000 tons (Wild et al. 1993) while actually no are available recent data on the amount of PCP produced globally (Van der Zande 2010).

Table 1. Chemical identity of PCP *.

Characteristic	Pentachlorophenol
Synonym(s)	PCP; penchlorol; penta; pentachlorophenate; 2,3,4,5,6-pentachlorophenol
Chemical formula	C_6HCl_5O
Chemical structure	
Registered trade name(s)	Chlon; Dowicide 7; Dowicide; EC-7; Dura Treet II; EP 30; Fungifen; Woodtreat; Permasan; Liroprem; Penta Concentrate; Penta Ready; Penta WR
Identification numbers:	
EPA Hazardous Waste	U242; FO27

* This informations were obtained from Hazardous Substances Data Bank

Available data: <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~7gat8x:1>

It is likely that the trend of use and production world level has decreased, because the PCP-demand has declined and for the stringent limiting actions taken in many countries over the years. In United States of America (USA) has been classified as a priority pollutant by the US Environmental Protection Agency and its use was restricted to certified applicators (EPA 1988). In 1975 the USA produced 23.600 tons of PCP while the recorded production in 2002 is about 4500 tons. Its use was 19.000 tons in 1984; 17.300 in 1985; 14.515 in 1986; 8.300 tons in 1996 and 5.000 tons in 2002 (WHO 2003; Van der Zande 2010). In Canada all product containing PCP were voluntarily withdraw in the 1990, remaining PCP uses only for heavy duty wood preservation and ground line remedial treatments of utility poles. In the 1992 the use of PCP was subject to regulation (CCME 1997). In China the annual production of PCP fell of the 70% the in twenty years among 1980-2000 (Tan and Zhang 2008). In Japan, from 1962 to 1970, was produced approximately 15.000 tons/year, but in the 1971 the PCP was banned due to its enormous danger (Crosby 1981). India is one of the few remaining countries still abundantly engaged in the large scale manufacture, use and export PCP (Abhilash and Singh 2009). In Australia, although in the last century PCP has been extensively used for timber preservation, now there are no longer any pesticides utilizing PCP registered for use (APVMA 2006). In New Zealand an estimated of 5000 tonnes of sodium pentachlorophenol (Na-PCP) was used over a period of forty years. The use of PCP in the timber industry ceased in 1988, and it was withdrawn from sale in 1991. Perhaps 40% of the PCP used in New Zealand was used at Weipa, amounting to an estimated of 2,000 tonnes of PCP and Na-PCP between the 1950's and 1988. There are several hundred sawmill and treatment sites where PCP was used in both the North and South Islands of New Zealand (Yu and Shepherd, 1997). In Europe the production of PCP ceased in 1992. Before that time, it was produced in many countries as Poland, Germany, Netherlands, Denmark, Switzerland, United Kingdom, Spain and France (Van der Zande 2010).

Table 2. Physical and Chemical Properties of PCP *.

Property	Pentachlorophenol
Molecular weight	266.35
Color	Colorless or white (pure); dark gray to brown (crude product)
Physical state	Crystalline solid (pure); pellets or powder (crude product)
Melting point	190 °C
Boiling point	309 – 310 °C
Density	1.978 g mL ⁻¹ at 22 EC
Solubility:	
Water	14 mg L ⁻¹ at 20 °C
Organic solvent(s)	Very soluble in alcohol and ether; soluble in benzene; slightly soluble in cold petroleum ether
Partition coefficients:	
Log Kow	5.01
Log Koc	4.5
Vapor pressure at 25 °C	0.00011 mmHg
Photolysis	t _{1/2} = 48 h
Henry's law constant at 25 °C	3.4x10 ⁻⁶ atm-m ³ mol ⁻¹

* This informations were obtained from Hazardous Substances Data Bank

Available data: <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~7gat8x:1>

After PCP production was stopped, it has been imported to the European market from the USA and southeast Asia. However in Europe the total consumption decreased of the 82% in ten years among 1985-1995 (Muir and Eduljee 1999). There are no complete data available of PCP use and production for South America and Africa.

1.3. Toxicity of PCP

PCP is a xenobiotic compound that in the animals is capable to generate inhibition of oxidative phosphorylation (Montiel et al. 2004), loss of cellular lytic function (Taylor et al. 2005), alter cell membrane functionality (McAllister et al. 1996), fetotoxicity, embrotoxicity, to bind to various hormone reception, endocrinopathy (Hong et al. 2010), DNA damage (Witte et al. 1985; Sai-Kato et al. 1995; Ma et al. 2010), disorders to cardiovascular system, blood, liver, kidney, immune system, central nervous system (ATSDR 2001), carcinogenicity, teratogenicity and eventually death may occur (Crosby 1981). Specifically in mammals PCP generates high body temperature, sweating, dehydration, rapid pulse, early coma, visceral hemorrhage, inactivation of respiratory enzymes, inhibition of glycolytic pathways and damage to mitochondrial structure (Gautam et al. 2003).

In plant it generate an inhibitory influence on seed germination and plant growth (Marihal et al. 2009), decrease in levels of biosynthesis of photosynthetic pigments, soluble protein (Hong et al. 2010), produce an inhibition of the formation of ATP by uncoupling oxidative phosphorylation, an variation in the speed of the propagation of electrical pulses (Volkov et al. 2000), can cause alterations in the pollen tube ultrastructure (Kandasamy and Kristen 1987), an change in the activity of antioxidative system and lipid peroxidation in leaves (Michałowicz et al. 2010).

Finally even on microorganisms the PCP expresses its toxicity (Watanabe 1978; Pignatello et al. 1983). PCP can influences not only in general microorganisms

community but also more specifically cellular process, cyclic changes in morphology, lipid membrane components, biomass growth, enzymatic activity, sporulation and reproduction capacity (Watanabe 1978; Bajpaia and Banerjib 1992). It is important to remember that was used just for to protect wood and tissues from attack by fungi and bacteria (Gadd 2001).

1.4. Environmental contamination by PCP

At any rate high PCP levels concentration were found near the contamination source (Bajpaia and Banerjib 1992; McAllister et al. 1996) but also a long distance (ATSDR 2001). In many cases the concentration levels were found of several thousand mg PCP kg⁻¹ (Bajpaia and Banerjib 1992).

The disposal of product containing PCP requires special actions. Many countries may have specific regulations, guidelines or recommendations for the management and disposal in discarded PCP but the most common disposal processes provide is reuse.

High PCP use has produced high levels of contamination and the compound was found in all environmental compartments (Field and Sierra-Alvarez 2008; Hong et al. 2010) and bioaccumulated within all trophic levels (Singh 2006). In fact its presence has been detected in air, soil, lakes, rivers, basins, snow, sediments, rainwater, drinking water, aquatic organisms, plants, fungi, bacteria, eggs, in mammals milk, blood, adipose tissue and urine (ATSDR 2001; Singh 2006). In atmosphere the PCP is released by volatilization and its transformation can be obtained by photolysis for slow free radical oxidation in an estimated mean period of approximately 2 months (ATSDR 2001). Instead in water PCP is subject to biotransformation and photodecomposition above all on the surface (Castillo and Bárcenas 1998). Finally in soil, the two main processes for the PCP removal are adsorption and degradation. They are dependent from all physical, chemical and biological soil properties (McAllister et al. 1996). Volatilization and

photolysis do not appear to be important processes in the transport and transformation of PCP in soil. In water and soil PCP is released for spillage or by leaching of contaminated material and its residual mean life is respectively of six and seven months (Buyuksonmez et al. 1999).

When the product is no longer suitable for reuse due to its size or condition, the remediation methods by PCP most commonly used are: adsorption with activated carbon, burned in an approved secure area (chemical landfill), treated in air stripping (based on the phase transfer and non-degradation of the compound) or with electrokinetic technology (limited by the solubility and desorption of organic pollutants in soil) and closed in sealed containers (ATSDR 2001; EPA 2008). Washed out with chemical products or incineration at high temperatures for a long time (EPA 2008). Especially the latter is not widely accepted due to the toxicity of the ash and for emissions of other substances very dangerous (Zhang and Qiao 2002). Depletion of PCP from can occur by evaporation, photochemical decomposition, water or oil leaching (McAllister et al. 1996) and finally for biological degradation (Bajpaia and Banerjib 1992; McAllister et al. 1996; Field and Sierra-Alvarez 2008; Rubilar et al. 2008).

1.5. Biological remediation methods

Biological remediation, or bioremediation, is an expanding area of environmental biotechnology and may simply be considered to be the application of biological process to treatment of pollution. The metabolic versatility of some organisms underpins practically all bioremediation applications and most work to date has concentrated on organic pollutants, although the range of substances which can be transformed or detoxified by some organism includes solid and liquid wastes, natural materials and inorganic pollutants such as toxic metals and metalloids.

Bioremediation include the use of microorganisms, for instance fungi, bacteria or other microbes, or in some cases also plants (phytoremediation), for to degrade, adsorpt and mineralize the contaminants found in soil and water, converting them to other intermediate or final products. Though it usually uses bacteria and fungi to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their aerobic or anaerobic metabolism. Usually, aerobic biodegradation is much more efficient than the anaerobic process and it is widely used in relation to the chemical nature of the contaminant. Both processes can be applied in series to reduce the complexity and toxicity of the contaminant. Biodegradation of a compound is often a result of the actions of multiple organisms. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. The control and optimization of bioremediation processes is a complex system of many factors. These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients) (Vidali 2001). Bioremediation technologies can be broadly classified as in situ (bioventing, biosparging, bioaugmentation and biostimulation) and ex situ (landfarming, biopiles and bioreactors). In situ techniques involve treatment of the contaminated material in place. By contrast, ex situ techniques are those treatments which involve the physical removal of the contaminated material for treatment (Vidali 2001).

1.6. Microbial degradation of PCP

One area that has potential for removal of PCP is microbial degradation. Microorganisms play an important role in the field of environmental science by degrading and transforming PCP into non-toxic or less toxic forms. Naturally how completely and efficiently PCP degradation occurs depends by microorganisms and the environmental conditions.

Aerobic microorganisms able to PCP biodegradation have been isolated in a variety of environments: industrial sewage, activated sewage sludge, soils and freshwater sediments (McAllister et al. 1996; Gadd 2001; Singh 2006). Aerobic PCP degradation by mixed microbial cultures is important since most PCP contaminated sites are surface soil or sediments which may support growth and activity of aerobic consortia. PCP degradation can occur by the combined efforts of microorganisms in these consortia. Some pure cultures of bacteria and fungi isolated from PCP contaminated sites are capable to mineralize high concentration of PCP ($>200 \text{ mg L}^{-1}$) as evidenced in McAllister et al. (1996) and Field and Sierra-Alvarez (2008).

Anaerobic microorganisms capable to degrade PCP are involved in numerous researches of critical importance. Contaminated area with PCP include anoxic environments as such as soils, water, sediments and industrial sludge (McAllister et al. 1996; Field and Sierra-Alvarez 2008). Anaerobic environments contain microbial consortia involved in methanogenesis and sulphate reduction are important to evaluate the effect of PCP on these anaerobic process.

In general, aerobic microorganisms have a greater ability to mineralize higher PCP concentrations than do anaerobic microorganisms. In addition, more efficient mineralization of PCP occurs by axenic cultures of bacteria than by fungi (McAllister et al. 1996; Field and Sierra-Alvarez 2008).

There are numerous studies that focus research efforts on degradation of PCP by pure and mixed cultures of aerobic and anaerobic microorganisms. Conditions that inhibit and enhance degradation, and pathways, intermediates and enzyme systems implicated essentially in PCP degradation especially by bacteria and fungi (Bajpaia and Banerjib 1992; McAllister et al. 1996; Gadd 2001; Field and Sierra-Alvarez 2008; Rubilar et al. 2008).

1.7. Research objectives

The current study, part of a wider bioremediation project, is a long-term evaluation of the remediation ability of naturally selected microorganisms versus PCP in laboratory-scale experiments. The main aims of this thesis were:

1. to define PCP sensitivity, adsorption and degradation of *Byssoschlamys nivea* (Westling 1909), *Scopulariopsis brumptii* (Samson and Klopotek 1972) and *Anthracoophyllum discolor* (Mont. Singer 1951) in microbiological culture media;
2. to test *B. nivea* and *S. brumptii* as antagonist against two Oomycetes: *Phytophthora cinnamomi* and *Phytophthora cambivora*;
3. to evaluate the response of an agricultural soil to PCP, with or without compost (biostimulation) and *B. nivea* and *S. brumptii* (bioaugmentation), in terms of effects of the contaminants on the main chemical, biochemical and biological soil properties.

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Armillaria mellea

2. A comprehensive overview of bacteria and fungi used for pentachlorophenol biodegradation

2.1. Abstract

Pentachlorophenol (PCP) has been a pollutant worldwide and extremely dangerous due to the high toxicity towards all organisms. It has been introduced into the environment mainly as a wood preservative, biocides and for the bleaching of paper or tissues. The use of PCP indiscriminate has led to the contamination of water and soil systems. Many countries have specific regulations, guidelines or procedures for the management and disposal of PCP but the most common methods are: adsorption with activated carbon, incineration in an approved and secure area, closed in sealed containers and biological degradation. PCP depletion can occur by abiotic processes such as: absorption, volatilization and photo degradation. In biotic degradation, one of the main studies focused on remediation utilizing the plants, animals and microbial community's. Aerobic and anaerobic microorganisms can degrade PCP under a variety of conditions and at different PCP concentrations. Bacterial strains as *Pseudomonas* sp., *Sphingomonas* sp., *Arthrobacter* sp., *Mycobacterium* sp., *Flavobacterium* sp., *Serratia* sp. and *Bacillus* sp., and fungal cultures as *Trametes* sp., *Phanerochaete* sp., *Anthracoophyllum* sp., *Armillaria* sp., *Bjerkandera* sp., *Ganoderma* sp., *Lentinula* sp., *Penicillium* sp., *Trichoderma* sp., *Rhizopus* sp. and *Plerotus* sp. showed various rates and extent of PCP degradation. This review focuses on PCP degradation by various aerobic and anaerobic microorganisms with emphases on the biological and chemical aspects. Furthermore we will analyze intermediate products, processes and enzyme tools involved in the degradation of PCP in different environmental conditions and at various PCP concentrations.

2.2. Abbreviations

Pentachlorophenol	PCP
Pentachloronanisole	PCA
Phenol	PH
Chlorophenol	CP
Dichlorophenol	DCP
Trichlorophenol	TCP
Tetrachlorophenol	TeCP
6-chlorohydroxyquinol	CHYQ
Chlorohydroquinone	CHQ
Dichlorohydroquinone	DCHQ
Trichlorohydroquinone	TriCHQ
Tetrachlorohydroquinone	TCHQ
2-chloro-1,4-benzenediol	DECB
2,6-dichloro-1,4-benzoquinone	DCBQ
Tetrachloro-1,4-benzoquinone	TeCBQ
Tetrachlorocatechol	TCC

2.3. Introduction

Pentachlorophenol (PCP) is an artificial semivolatile organochlorine compound abundantly used in as biocide low cost in agricultural and industrial purposes. High PCP use has produced high levels of environmental contamination. In fact its presence has been detected in air, soil, lakes, rivers, basins, snow, sediments, rainwater, drinking water, aquatic organisms, plants, fungi, bacteria, eggs, in mammals milk, blood, adipose tissue and urine (ATSDR 2001; EPA 2008).

To remove PCP from environment, several commonly methods are used: adsorption with activated carbon, burned in an approved secure area (chemical landfill), treated in air stripping (based on the phase transfer and non-degradation of the compound) or with electrokinetic technology (limited by the solubility and desorption of organic pollutants in soil) and closed in sealed containers (ATSDR 2001; EPA 2008). Washed out with chemical products or incineration at high temperatures for a long time (EPA

2008). Especially the latter is not widely accepted due to the toxicity of the ash and for emissions of other substances very dangerous (Zhang and Qiao 2002). A further low-cost methods to removal PCP is biological depletion such as microbial degradation (McAllister et al. 1996; Gadd 2001; Singh 2006; Rubilar et al. 2008; Field and Sierra-Alvarez, 2008; Juwarkar et al. 2010).

PCP degradation is a process that can be completed through three ways: oxygenolysis, hydroxylation or reductive dehalogenation (McAllister et al. 1996 ; Field and Sierra-Alvarez 2008). PCP depletion can occur by abiotic processes such as: absorption (Cea et al. 2005), volatilization (Crosby 1981) and photo degradation (Malato et al. 1998; Czaplicka 2006). In biotic degradation, the main studies focused on remediation by plant (Mills et al. 2006; Eapen et al. 2007), animal (Crosby 1981) and the microbial community (McAllister et al. 1996; Gadd 2001; Pointing 2001; Crawford et al. 2007; Field and Sierra-Alvarez 2008; Rubilar et al. 2008; Juwarkar et al. 2010).

Several microorganisms showed excellent ability at tolerating and removing PCP, a few examples are as follow: to avoid the toxicity of PCP by excluding it from the cell, by converting it to a non-toxic compound or by using PCP as the sole source of carbon (McAllister 1996). The capacity of PCP to transform into less toxic products, depends on environmental conditions including, water content (Seech et al. 1991), temperature (Valo et al. 1985), pH level and the organic matter (Cea et al. 2005), humic substances (Rüttimann-Johnson and Lamar 1997), oxygen and electron acceptors (D'Angelo and Reddy 2000).

Under anaerobic conditions, bacteria can transform PCP with reductive dehalogenation, where the chlorine atoms are sequentially replaced by hydrogen atoms until it is completely transformed into phenol (PH), benzoate, acetate, carbon dioxide and methane (Mohn and Tiedje 1992). Reductive dechlorination has been observed in many soils, sediments and sewage sludge (D'Angelo and Reddy 2000). Mikesell and

Boyd (1986) showed the complete reductive dechlorination of PCP by combined activities of indigenous bacteria. Fungi and bacteria can transform PCP by incorporating one or two oxygen atoms from the diatomic O_2 using the structure of the contaminant in an aerobic condition through the process of oxygenase. This process allows the destruction of the aromatic ring and the subsequent formation of CO_2 through a slow aerobic transformation; especially for the highly chlorinated compounds such as PCP (Reddy and Gold 2000). This is due to the fact that the aromatic ring is deficient in electrons and less susceptible to electrophilic attack by O_2 (Sahm et al. 1986). On the other hand, this may also be done by means of hydroxylation reactions which convert PCP into other compounds such as tetrachlorohydroquinone (TCHQ) (Xun and Orser 1991; Crawford et al. 2007; Xun et al. 2010) by replacing the chlorine atom with a hydroxide, converting the PCP into intermediate products (Vijay et al. 2000; Crawford et al. 2007; Xun et al. 2010). Other processes for the degradation of PCP, using either fungi or bacteria, can be achieved through methylation (McAllister et al. 1996; Gadd 2001). It occurs mainly for co-metabolism which is the particular reaction where specific enzymatic reactions are involved but not precisely targeted for this function. In fact many fungi have been shown to detoxify PCP by methylation using a specific lignin-degrading system, existing to serve other functions such as degradation of wood components such as lignin and cellulose (McAllister et al. 1996; Gadd 2001; Rubilar et al. 2008). Using reactions catalyzed by PH-oxidases such as laccases and peroxidases, fungi are able to make the primary PCP transformation into pentachloroanisole (PCA) (McAllister et al. 1996; Gadd 2001; Singh 2006; Rubilar et al. 2008). PCA is a less toxic form of PCP and because it is a compound with a more lipophilic composition it may be quickly bio accumulated (McAllister et al. 1996). The last process to remove PCP exploits the adsorption to biomass of fungi and bacteria (living or death). It has been found that some microbial cultures have a particular affinity for binding to PCP (Ahmaruzzam 2008; Rubilar et al. 2012) i.e. the adsorbing take advantage of the

attraction charge between PCP and microorganisms biomass. The principal degradation pathways, genes and associated enzymes involved in the detoxification mechanism against recalcitrant organic compounds like PCP for bacteria and fungi, have been widely studied especially in recent original paper and historical review (Crawford et al. 2007; Rubilar et al. 2008; Xun et al. 2010; Carvalho et al. 2011; Yadid et al. 2013; Copley et al. 2013).

However, numerous studies have demonstrated that under aerobic conditions, PCP can be efficiently reduced until a complete mineralization (Reddy and Gold 2000; Pointing 2001; Leontievsky et al. 2002; Walter et al. 2004; Crawford et al. 2007; Field and Sierra-Alvarez 2008; Rubilar et al. 2008; Xun et al. 2010).

In this paper we will focus our attention on the degradation of PCP by bacteria and fungi, analyzing primarily the species for a biological and chemical profile. In a second moment we exploring intermediate products, processes and enzyme tools involved in PCP degradation in microbiological culture media, soils, sludge and sediments in different environment conditions following the extensive review of McAllister et al. (1996).

2.4. PCP degradation by bacteria

In recent years, many bacterial strains isolated from every medium (soil, water, plant and animal) have been found useful in playing an important role in the tolerance, degradation and mineralization of PCP (McAllister et al. 1996; Field and Sierra-Alvarez 2008). While tolerance to PCP is a one of the main variables and can be a good starting point in the process of selecting useful strains for PCP degradation, the more interesting and, albeit, important aspect is the capacity of the microorganisms to degrade and mineralize PCP to CO_2 , Cl^- and H_2O (Crawford et al. 2007; Xun et al. 2010). When considering all bacteria, the genus most recently studied that may have

the best possible potential in regards to bioremediation works are: *Pseudomonas* sp., *Flavobacterium* sp., *Nocardioides* sp., *Novosphingobium* sp., *Desulfitobacterium* sp., *Mycobacterium* sp., *Sphingomonas* sp., *Kokuria* sp., *Bacillus* sp., *Serratia* sp. and *Acinetobacter* sp. (Table 3). All these bacteria may be isolated from different substrate.

2.4.1. The genus *Acinetobacter*

Acinetobacter is a genus of Gram-negative bacteria. Within this genus there are a diverse group of organism that range from human pathogens to environment. This bacterium is non-motile, containing multiple compounds that exhibit an oxidative capacity to arrive at final mineralization (e.g. CPs) (see McAllister et al. 1996). These characteristics are used in various biotechnological applications, including bioremediation.

Acinetobacter sp. ISTPCP-3, was isolated by Sharma et al. (2009), was capable of degrading PCP. An optimum growth condition for the bacterial strains in the presence of PCP was investigated with varying pH levels, initial PCP concentrations and temperatures. The results indicated elevated PCP degradation between pH levels of 6.5 and 7.5. The optimum condition for the maximum degradation of PCP was at pH level 7.0 degrading 95% of 50 mg L⁻¹ PCP. The bacterial strains were able to completely degrade PCP at all concentrations lower than 100 mg L⁻¹ within 48 h. At 200 mg L⁻¹ PCP, the degradation was incomplete. The optimum growth temperature for the strain was at 30°C. It degraded 50 mg L⁻¹ PCP after only 24 h. *Acinetobacter* sp. ISTPCP-3 was able to utilize PCP through an oxidative process with ortho ring-cleavage producing the formation of 2,3,5,6-TCHQ and 2-chloro-1,4-benzenediol (DCBE). Sharma and Thakur (2008) isolated two bacterial strains identified as *Escherichia coli* PCP1 and *Acinetobacter* sp. PCP3. The ability of these two bacterial strains to effectively degrade PCP was observed with an emphasis on the growth and utilization

of PCP. During the experiment the parameters were 96 h at 30°C, with pH levels between 7.2-7.4, all being in a mineral medium supplemented with 100 mg L⁻¹ PCP. Utilization of PCP was higher in *Acinetobacter* sp. PCP3 which exemplified the capacity to utilize PCP, more than 20% within 6 h, while *E. coli* PCP1 utilized only 10%. However, the most significant result observed in this study was the utilization of more than 80% of PCP by *Acinetobacter* sp. PCP3. After 96 h followed only by *E. coli* PCP1 that utilized almost 60%. The release of intermediate products such as TCHQ, 2, 3, 4, 6-TeCP and DCBE were most present in *Acinetobacter* sp. PCP3 rather than *E. coli*.

2.4.2. The genus *Bacillus*

Bacillus is another genus capable of PCP degradation under aerobic conditions (Field and Sierra-Alvarez 2008). Bacteria belonging to *Bacillus* sp. have great skills in metabolizing various industrial pollutants, many of which are complex organic compounds. It is a model organism for laboratory and field studies and is one of the best understood bacteria in terms of ecology and biology. *Bacillus* strains can make a pivotal impact on residual life of PCP.

Karn et al (2010b) isolated by secondary pulp and paper industry sludge, *Bacillus* sp. Strains were capable of PCP bioremediation. The strains were isolated and identified as: *Bacillus megaterium* CL3, *Bacillus pumilus* CL5 and *Bacillus thuringensis* CL11. The microorganisms were used in PCP degradation in a mineral medium where the initial PCP concentration was 100 mg L⁻¹. The effect of PCP on the growth and degrading ability of *B. megaterium* CL3, *B. pumilus* CL5 and *B. thuringensis* CL11 were also examined at different concentrations: 50, 100, 200, 400, and 600 mg L⁻¹ PCP. The degradation capability test was executed with varying parameters such as pH level and temperature. In mineral mediums all three isolates were able to grow and utilize

PCP. Degradation of PCP increased with time for all of the isolates. The strains were able to completely remove PCP from the medium within 168 h. All these isolates were able to degrade more than 90% of PCP at 400 mg L⁻¹. Strains *B. megaterium* CL3 and *B. thuringensis* CL11 were able to remove up to 80% when grown at 600 mg L⁻¹ PCP, compared to that of strain *B. pumilus* CL5, which was able to remove 91% of PCP at the same concentration. These results show that all *Bacillus* isolates have the ability to degrade PCP at high concentrations. All the isolates removed 90% of PCP between pH levels of 7.5 and 8.5 while PCP removal efficiency significantly decreased when the initial pH level of the medium was 9.5. When testing diverse temperatures the removal of PCP was less efficient at 25 °C than at 30 °C or 37 °C for the three bacteria species. Tripathi et al. (2011) collected 42 isolates from treated tannery effluent in India, one being, identified as *Bacillus* sp. was tolerant at 500 µg ml⁻¹ PCP. The results clearly indicate that there was a concomitant increase in bacterial growth during 0–48 h with as much as 56% of PCP was simultaneously biodegraded after 48 h of incubation. Singh et al. (2009) isolated and studied *Bacillus cereus* DQ002384 in regards to PCP degradation, individually and in mixed cultures with two *Serratia marcescens* strains, AY927692 and DQ002385. In an optimized growth condition, mixed cultures were found to be able to degrade up to 93% of PCP at concentrations of 300 mg L⁻¹, on the other hand, when used individually *B. cereus* DQ002384 only degraded 62.75% of PCP. Many intermediate products of PCP degradation were detected, such as TCHQ. Chandra et al. (2006) isolated a PCP degrading bacterial strain known as *B. cereus* ITRC S₆. The degradation and bacterial growth were performed in flasks containing 300 mg L⁻¹PCP and 1% glucose. The bacteria showed good tolerance and growth with PCP reaching the stationary phase after only 144 h of incubation. But, it showed no growth in absence of glucose; thus, indicating that PCP degradation was the resultant of co-metabolism. *Bacillus cereus* ITRC S₆ degraded about 62.75% of PCP during 168 h of incubation. In any case *B. cereus* ITRC S₆, alone or in mixed cultures was used by

Chandra et al. (2009) for the treatment of pulp and paper mill effluent with contaminated levels of $50.3 \pm 1 \text{ mg L}^{-1}$ PCP, with all studies being conducted within 168 h. The PCP reductions from the pulp and paper mill effluent using the bacterial strains were quite impressive, emphasizing their ability to synergic interaction. In fact, *B. cereus* ITRC S₆ when used in mixed cultures degraded 90 and 100%. In both cases the capacity to degrade PCP was very high. Only when there was a synergic interaction was the PCP completely degraded. When using the effluent samples to confirm the ability to degrade PCP by the varied bacteria strains the products 2, 4, 6- TCP, TCHQ, ChLO and TriCH were found.

2.4.3. The genus *Desulfitobacterium*

Desulfitobacterium genus was discovered in the last decade and can dehalogenate organic compounds through mechanisms of reductive dehalogenation. They are versatile microorganisms, strictly anaerobic bacteria, that can be used with a wide variety of electron acceptors, such as nitrate, sulfite, metals, humic acids, and man-made or naturally occurring halogenated organic compounds (van Elsas et al. 1997).

Tartakovsky et al. (1999) studied the PCP degradation capacity of *Desulfitobacterium frappieri* PCP-1, adding it to a mixed bacteria community in an anaerobic bioreactor. There were two initial concentrations used: 1 mg L^{-1} and 100 mg L^{-1} PCP. While the incubation time was: 30 and 60 days. PCP removal efficiency was 99% and the dechlorination efficiency was 90.5%. PH and TCP were observed as dechlorination intermediate products. *D. frappieri* PCP-1 transformed PCP to TCP, and TCP to PH followed by PH-mineralization, which was caused most likely by indigenous microorganisms. Lanthier et al. (2005) developed a PCP-degrading, methanogenic fixed-film reactor, by using broken granular sludge. This consortium acclimated to increasing concentrations of PCP. After 225 days of acclimation, the reactor was

performing at a very high level. They reached a PCP-degrading rate of $1.173 \mu\text{M day}^{-1}$, with a PCP degradation efficiency of approximately 60%. Only TCP was observed as an intermediate product. PCR species-specific primers highlighted a significant presence of *Desulfitobacterium hafniense* in the biofilm test during the reactor acclimation phase. *D. hafniense* cells were scattered in the biofilm and they accounted for 19% of the community. These results suggest that the presence of PCP-dehalogenating *D. hafniense* in the biofilm was crucial for the performance of the reactor.

2.4.4. The genus *Flavobacterium*

Flavobacterium sp. are generally communal bacteria rod-shaped Gram-negative that live in soil and water (van Elsas et al. 1997). This genus include economically disastrous animal pathogens (especially in freshwater fish) and environmental bacteria. Some species of *Flavobacterium* are capable of degrading PCP and other similar compounds (McAllister et al. 1996).

Pfender et al. (1997) used *Flavobacterium* sp. ATCC53874 in a laboratory-scale bioremediation in a soil microcosm amended with 125 ppm PCP. Over 50% of the available PCP was quickly mineralized from soil by *Flavobacterium* sp. ATCC53874 within 4 days. While after 42 days of incubation, about 65% of PCP was mineralized. *Flavobacterium* sp. ATCC 21918 in a mixed culture (in combination with *Arthrobacter* sp. ATCC 33790) was used by Pu and Cutright (2007) to evaluate the PCP biodegradation in two different field soils, from Columbia (CO) and New Mexico (NM). The soils were incubated for 56 days. In the CO soil, with the presence of *Flavobacterium* sp. ATCC 21918 as well as the mixed culture, the initial concentrations were at $539 \text{ mg PCP kg}^{-1}$ soil. After 56 days in CO soil the biodegradation efficiencies were 12% and 25% for *Flavobacterium* sp. ATCC 21918

and the mixed culture. In NM soil, the initial concentration was 262.26 mg PCP kg⁻¹ soil for *Flavobacterium* sp. ATCC 21918 also in the mixed culture. The biodegradation in soil was 79.2% and 98.2% for *Flavobacterium* sp. ATCC 21918 and the mixed culture. Finally in the NM soil a bio stimulation experiment was carried out using a nutrient solution but in every case PCP degradation was 60%.

2.4.5. The genus *Kocuria*

Kocuria, previously classified into the genus *Micrococcus* (which are closely related phylogenetically but differ in some chemotaxonomic properties) is a common bacteria which is widespread. This organism is an strictly aerobic, Gram-positive coccus occurring in tetrads with a majority of strains being non-pathogenic (van Elsas et al. 1997).

Karn et al. (2011) used *Kocuria* sp. CL2 in the PCP degradation in a mineral medium batch culture at 100 mg L⁻¹ PCP. During the 168 h. *Kocuria* sp. CL2 utilized 55%, 95% and 100% after 24, 96 and 168 h of incubation. During the course of the bacterial treatment, PCP was mineralized and the liberation of an inorganic chloride ion into the culture medium was observed. The concentration of the chloride ion increased as the degradation of PCP continued. This study showed that the removal efficiency of PCP by *Kocuria* sp. CL2 to be very effective and can be used in the degradation of PCP which is contained in pulp and paper mill waste often times found in the environment. In the same work *Kocuria* sp. CL2 was used also in the PCP degradation in sludge. Flasks were incubated for 336 h. Tolerance and degradation tests was carried out at 100 mg L⁻¹ PCP. The *Kocuria* sp. CL2 strain was capable of mineralizing PCP. It was able to remove up to 58.64% PCP from the sludge.

2.4.6. The genus *Mycobacterium*

The bacteria belonging to this genus are aerobic, non-motile (except for only one species which has been shown to be motile) and Gram-positive microorganisms that includes pathogens known to cause serious diseases in mammals. The mycobacteria have been observed to grow in a fungus-like when cultured in liquid medium i.e. this alludes the suffix *myco*. Thanks to enzymatic tools, this genera is widely used also in bioremediation studies.

A strain widely used in PCP degradation, mostly in soil, is *Mycobacterium chlorophenolicum* (*Rhodococcus chlorophenolicus*). It is a Gram-positive bacterium non-motile well-performing at biodegrading (van Elsas et al. 1997). Furthermore *M. chlorophenolicum* in some cases can exhibit cyclic change in morphology from coccus to rod in the presence of a contaminant like PCP (Häggblom et al. 1998). Miethling and Karlson (1996) studied mineralization of 30 and 100 mg PCP Kg⁻¹ soil using also *Mycobacterium chlorophenolicum* PCP1. They compared the activity of soil without inoculation, determining its natural capacity of PCP mineralization. Non inoculated soil completely mineralized 30 mg PCP Kg⁻¹ soil within 7 months, but showed little to no degradation activity at 100 mg PCP Kg⁻¹ soil in the same time period (less than 2%). At 30 mg PCP Kg⁻¹ soil, inoculated with *M. chlorophenolicum* PCP1 increased the mineralization slightly over what the indigenous bacterial activity produced. At 100 mg PCP Kg⁻¹ soil only 27% was mineralized within 7 and a half months. The mineralization of PCP in sterile and non-sterile soil microcosm with or without the addition of *M. chlorophenolicum* PCP-1 was examined by Combrisson and Jocteur Monrozier (1999). In this case the soil used in the study, never had a contamination of PCP. The soil microcosms were incubated with 22 mg PCP Kg⁻¹ soil for 60 days. Only 5% of the PCP was mineralized in the sterile soil with or without *M. chlorophenolicum* PCP-1. About 50% of PCP was mineralized in a non-sterile soil with or without the bacterium strains. These results suggest that the PCP was not easily accessible to *M.*

chlorophenolicura PCP-1 and that PCP mineralization could only occur if the operation was in a microbial consortium.

2.4.7. The genus *Pseudomonas*

Microorganisms widely studied are *Pseudomonas* sp., ubiquitous bacteria with good potential in bioremediation. There are many species of the genus *Pseudomonas* with the capability to use many chlorophenols i.e. Chlorophenol (CP), Dichlorophenol (DCP) and Trichlorophenol (TCP) as carbon and energy sources under aerobic conditions including PCP (McAllister et al. 1996; Field and Sierra-Alvarez 2008). Almost all strains of this genus that are analyzed in the following studies were isolated from PCP contaminated sites (soil, secondary sludge of pulp and paper mill, aquifer sediments, tannery effluent, groundwater) showing a good capacity of PCP tolerance and degradation at very high concentrations of contaminant. The most efficient strain of *Pseudomonas* sp. that was able to remove PCP was isolated by Lee et al. (1998) in Korea. The isolate *Pseudomonas* sp. Bu34 degraded almost 75% of 4000 mg L⁻¹ PCP after 57 days and about 90% of 1000 and 2000 mg L⁻¹ PCP during 30 days of incubation. Toxicity test (comparing acclimated and non-acclimated cells, where acclimated insinuates previous contact with PCP) showed in non-acclimated, the cell number of strain Bu34 decreased although within 24h increasing culture amount of PCP from 75 to 4000 mg L⁻¹. In the acclimated strain the toxic effect did not appear until concentrations of 1000 - 4000 mg L⁻¹ PCP. In fact, in acclimated experiments the number of cells of *Pseudomonas* sp. Bu34 considerably increased, achieving stationary phase within 10 days. Karn et al. (2010a) isolated *Pseudomonas stutzeri* CL7 that was able to utilize 90% of PCP at concentrations between 50 - 600 mg L⁻¹ PCP. More than 95% PCP degradation was recorded exceeding at 200 mg L⁻¹ PCP. The isolate completely mineralized PCP after 120 h of incubation showing good growth in relation to simultaneous liberation of chloride ions. The initial concentration of the chloride ion

was 200 mg L⁻¹ but during 160 h it reached values of 500 mg L⁻¹. Furthermore radial and biomass growth of *P. stutzeri* CL7 was significantly reduced increasing the concentration of PCP. Kao et al. (2005) isolated *Pseudomonas mendocina* NSYSU by analyzing its capacity to degrade PCP, changing pH levels and temperature. The results showed that PCP was rapidly removed after only 12 days at any of the following PCP concentrations: 20, 40, 80 and 100 mg L⁻¹ PCP. The concentration of 150 mg L⁻¹ PCP, demonstrated a complete depletion after 18 days. No PCP removal was detected at concentrations of 320 mg L⁻¹ PCP within the 20 days of incubation. The analysis indicated that the optimal capacity of degradation for *P. mendocina* NSYSU include the following conditions: slightly acidic (6 < pH < 7), aerobic and relatively moderate ambient temperature (20 °C < temperature < 30 °C). Finally, in microcosm experiments the following PCP degradation products were recovered: 2, 4, 6-TCP; 2, 4-DCP; 4-CP and 2-CP. Shah and Thakur (2002) tested *Pseudomonas fluorescens* TE3 in degradation test at a concentration of 100 mg L⁻¹ PCP. Bacterial strains grew in the PCP within the first 72 h, while later declined and degraded 72% of PCP after 96 h which resulted in the highest release of chloride. The degradation of PCP *P. fluorescens* TE3 was conducted by using the oxidative process as indicated by the accumulation of degradation products such as chlorohydroquinone (CHQ), dichlorohydroquinone (DCHQ) and TCHQ. All which are intermediary metabolites used by the bacterial strain. Finally *P. fluorescens* TE3 showed a greater capability to degrade all the intermediate compounds, but the maximum utilization was only 70% of TCHQ. Sharma and Thakur (2008) used *Pseudomonas aeruginosa* PCP2 in degradation of PCP monitoring growth and utilization of 100 mg L⁻¹ PCP. The isolate used more than 15% within 6 h and 65% in 96 h. Gautam et al. (2003) used *Pseudomonas* sp. IST103 in PCP degradation at 0.1 g L⁻¹. It utilized PCP continuously until a maximum of 70% after 96 h. The PCP utilization was also supported by chloride release and ring cleavage. Bacterial strains Gram-negative, identified as *Pseudomonas* sp. were used

from Yu and Ward (1996) in which degradation was monitored at 100 ppm PCP, individually and in mixed cultures with other bacteria (*Flavobacterium gleum* and *Agrobacterium radiobacter*). PCP degradation using only *Pseudomonas* sp. was 20%, while in the mixed culture was 80%. Nam et al. (2003), in Korea, isolated a new strain for PCP degradation in the family of *Pseudomonas*. It was identified as *Pseudomonas veronii* PH-05. The amount of PCP decreased in time, with a gradual increase in cell density. PCP's initial concentration of 0.3 mM decreased to 0.21 mM. About 30% of PCP was bio-transformed to metabolic intermediates such as tetrachlorocatechol (TCC). After 72 h of incubation, about 12% of PCP had been converted into TCC. A strain of *P. fluorescens* was found in South Africa by Lin et al. (2008) and was found to be able to degrade 200 μM PCP after only 4 days. Optimal PCP degradation conditions of *P. fluorescens* were at a pH level of 7 and a temperature of 30°C. The supplementation of 1% glucose stimulated the growth of the microorganism and enhanced the ability to utilize PCP from the effluent sample. The authors did not show values or percentages of the PCP that was degraded. In Finland, a contaminated site was found with 1 mg L^{-1} PCP, Männistö et al. (1999) isolated and tested seventeen bacteria strains for PCP degradation. From these bacteria, the authors isolated *Pseudomonas amygdali* K104. They evaluated the PCP degradation when the compound was alone or in a mixed solution which contained: 80% 2, 3, 4, 6-tetrachlorophenol (2, 3, 4, 6-TeCP) and approximately 20% PCP. The initial concentration of PCP was 2 mg L^{-1} , while the 2,3,4,6-TeCP was: in the first test 0.2 mg L^{-1} PCP. In second test only 1 mg L^{-1} PCP was used. About 60% of PCP was degraded while in mixed a compound with 2, 3, 4, 6-TeCP at both concentrations. When PCP was alone, *P. amygdali* K104 did not degrade the contaminate. Nakamura et al. (2004) used a particular genes encoding system to contrive PCP-degrading enzymes from *Sphingomonas chlorophenolicum* (Dai and Copley 2004). They introduced this gene into the chromosome of *Pseudomonas gladioli* M-2196, which achieved the

transformation of a strain with the ability to degrade PCP to a maximum concentration of 3 μl PCP. This strain degraded more than 80% PCP within 4 days. TCC was the metabolite in PCP degradation.

Using indigenous microorganisms, specifically *Pseudomonas* sp., and nutrient amendments (essentially N and P), Schmidt et al. (1999) evaluated the PCP degradation in four batch reactor test's. The study had the objectives of determining the rate and extent of PCP removal in conditions with unamended or amended mediums with N and P. The first phase of the experiment was conducted during 28 days of incubation with a PCP initial concentration of 0.405 mg L^{-1} . In the second phase, PCP degradation was evaluated after 32 days of incubation with a PCP initial concentration at 0.474 mg L^{-1} . In all cases, PCP decreased from the initial concentration up to <0.002 mg L^{-1} PCP with the exception of the abiotic control. The results that the test showed exhibited that fact that PCP removal it positively affected by the presence of N and P.

Gautam et al. (2003) isolated *Pseudomonas* sp. IST103 from the effluent sediment of paper mill in India. The strain was tested in two sets of soil microcosms containing 20 and 40% moisture, each having the following PCP concentrations: 0, 10, 100, 500, and 1000 mg L^{-1} . *Pseudomonas* sp. showed significant utilization of PCP, about 80%, with higher cell growth after 45 days, the highest being when PCP was applied up to levels of 100 mg L^{-1} and a concentration of 20% moisture. At 40% moisture about 70% of PCP was used. Inhibitory effects on the growth of the bacterial strain and PCP utilization were seen at 500 and 1000 mg L^{-1} PCP concentrations for both moistures. Finally, a qualitative analysis with HPLC showed that TCHQ was the metabolite of PCP degradation in soil microcosms *Pseudomonas* sp. SR3 was used by Pfender et al. (1997) in a laboratory-scale bioremediation of soil microcosm in a bottle amended with 175 ppm PCP. Over 50% of the available PCP was quickly mineralized from the soil within 4 days. After 42 days of incubation, about 65% of PCP was mineralized.

Nakamura et al. (2004) modified a chromosome of *Pseudomonas gladioli* M-2196 using a particular genes encoding system for PCP-degrading enzymes from *Sphingomonas chlorophenolicum* described by Dai and Copley (2004) transforming bacterium into a strain with the ability to degrade PCP in a soil microcosm. In the soil, the degradation capacity of PCP was much lower. In fact, in soil after 28 days of incubation the degradation was of only 10% with an initial concentration of 2.5 μl . Sejáková et al. (2009) studied PCP contaminated soils ability to degrade autochthonous microorganisms and the effects of bioaugmentation brought about by the bacterial strain *Pseudomonas testosteroni* CCM 7530. The biodegradation of PCP was performed in soil (Fluvisol, Chernozem, and Regosol) with the presence of *P. testosteroni* CCM 7530 as well as sans additional bioaugmentation. The biodegradation of PCP in soil was carried out under laboratory conditions in the real soil the initial PCP concentration was 10 and 100 mg PCP kg^{-1} soil. For each experiment, three sets of soil samples were used and analyzed after 7, 17 and 24 days. The soil samples with concentrations of 10 mg PCP kg^{-1} soil revealed higher degradation in comparison to the soil with 100 PCP mg kg^{-1} soil where the degradation was already observed within 7 days. The biodegradation of PCP in the bioaugmented soils evaluated after 24 days depended on other factors such as the addition of sorbent, initial PCP concentration, and above all the soil type. In bioaugmented Regosol and Fluvisol with a concentration of 10 mg PCP kg^{-1} soil, about 72–74% degradation was noted, while with Chernozem only 57%. Biodegradation of PCP in soils with 100 mg PCP kg^{-1} soil was remarkably lower (49% Regosol, 39% Chernozem and 34% Fluvisol). These PCP degradation values although interesting, were significantly lower in comparison to the same soils which were not inoculated with *P. testosteroni* CCM 7530 but amended with an organo-mineral complex or lignite. Bioaugmentation of the soil by external microorganisms with a PCP degradation capability did not ensure higher levels of pollutant being degraded. The success of decontamination and detoxification depends

on the soil type and more importantly on the amount of the organic soil matter. Vítková et al. (2011) studied the degradation capacity of autochthonous microorganisms and the effect on bioaugmentation by the bacterial strain *Pseudomonas testosteroni* CCM 7530 and the biostimulation with lignite in a PCP contaminated soil. The biodegradation experiments with PCP were performed in soils (Fluvisol, Chernozem, and Arenosol) with the presence of an inoculum of *P. testosterone* CCM 7530 also without additional bioaugmentation. The biodegradation of PCP in soil was carried out under laboratory conditions at concentrations of 10 and 100 mg PCP kg⁻¹ soil. For each experiment, three sets of soil samples were used and analyzed after 7, 14, and 21 days of incubation. The soil samples with concentrations of 10 mg PCP kg⁻¹ soil revealed higher degradation in comparison to soils with concentrations of 100 mg PCP kg⁻¹ soil, especially chernozem where the highest degradation was 78% in non-amended soil and 55% in lignite-amended soil. Biodegradation of PCP in bioaugmented soils were evaluated after 21 days. It depended on the soil type and the presence of lignite. The lignite exhibited significant improvement of degradation, about 20% in each soil type, except for chernozem and aerosol at 10 mg kg⁻¹ PCP, where the degradation was 29% and 55%. In general, the degradation of PCP was higher with the bioaugmentation conditions, mainly in chernozem and in lignite-amended soil. The degradation efficiency order is as follows: chernozem, fluvisol and arenosol. It can be concluded that lignite proved its protective effects, but for the most part only for the non-degrading autochthonous micro flora.

2.4.8. The genus *Serratia*

Serratia is a common genus of aerobic rod-shaped bacterium (optionally anaerobic) that has provided possible biotechnological approaches to clean up polluted environments contaminated by PCP in axenic condition (Abo-Amer 2011). The most

common species in the genus is *Serratia marcescens*, an human pathogens that may causes nosocomial infections.

Singh et al. (2009) confronted the synergistic PCP biodegradation of a microbial consortium composed by *Serratia marcescens* AY927692, *Serratia marcescens* DQ002385 and *Bacillus cereus* DQ002384 against the effectiveness of this bacterium alone. All experiments were carried out after 168 h in an optimized condition for growth of bacteria (at 30 ± 1 °C, pH 7.0 ± 0.2 , 120 r.p.m.) and at different environmental conditions, i.e., temperature (20, 30 and 37 °C), pH (6.0, 7.0 and 9.0) and aeration rate (50, 120 and 200 r.p.m.). The Initial concentration was 300 mg L^{-1} PCP. In an optimized condition for growth, the mixed culture was found to be able to degrade up to 93% of PCP, compared to a single *S. marcescens* strain AY927692 as well as DQ002385 which degraded PCP at percentages of 85.50% and 90.33%. Mixed cultures degraded 62.75% of PCP at 20 °C and 83.33% at 37°C; 70% at pH 6 and 75.16% at pH 9; 73.33% at 50 rpm and 91.63% at 200 rpm. However *S. marcescens* AY927692 was more skilled in PCP degradation in mixed cultures at following conditions: 50 rpm, 20 and 37°C, pH 6 and 9. Many intermediate products of PCP degradation were analyzed. The identification of TCHQ, CHYQ and 2,3,4,6-TeCP suggested that the degradation occurred through reductive dechlorination. The consortia showed better overall removal efficiencies than the single strains that used PCP as a carbon and energy source. Another strain of *Serratia marcescens* ITRC S₇ (Singh et al. 2007) was used in PCP tolerance and degradation experiments. It was found to be able to degrade up to 90.33% at 300 mg L^{-1} PCP with a simultaneous release of a chloride ion. Bacterial dechlorination occurred in mineral mediums when in the presence of glucose as an additional carbon and energy source, within 168 h of incubation. In absence of glucose the bacterium was unable to utilize PCP, indicating the process of co-metabolism. Finally the metabolites obtained for degradation of PCP were TCHQ and 6-chlorohydroquinol. *Serratia marcescens* ITRC S₉ and a mixed

culture with *Bacillus* sp. ITRC S₈ were used by Singh et al. (2008) in experiments dealing with PCP degradation of pulp paper mill effluent collected in India containing 50.31 mg L⁻¹ PCP. The degradation studies were performed in batch cultures composed from pulp and paper mill effluent samples, 1% glucose, 0.5% peptone and bacteria being individual or in a combination. Mixed cultures degrade PCP up to 94% with the simultaneous release of a chloride ion which was limited at the level of 1200 mg L⁻¹ after 168 h, emphasizing bacterial dechlorination in the medium. In same time span as well as, individually, the bacteria strains released chloride ions below 800 mg L⁻¹. Furthermore, in mixed culture the strains showed a growth and degradation of PCP at higher efficiencies than when alone. The final analysis with high performance liquid chromatography (HPLC) of pulp paper mill effluent degradation products showed the formation of 2-CP and TCHQ. From pulp and paper mill effluent sludge samples, collected in India, Chandra et al. (2006) isolated *Serratia marcescens* ITRC S₉. PCP degradation and growth exams were tested at 300 mg L⁻¹ PCP and 1% glucose. *S. marcescens* ITRC S₉ showed tolerance and growth with PCP reaching the stationary phase after 144 h of incubation. In addition, the bacterium strain did not grow in the absence of glucose; thus, it indicated that PCP degradation is the result of co-metabolism. *S. marcescens* ITRC S₉ degraded 86.6% of PCP degradation after 168 h of incubation. The PCP-degrading bacterial strains, *S. marcescens* ITRC S₇ and mixed cultures with *Bacillus cereus* ITRC S₆ were used by Chandra et al. (2009) for the treatment of pulp and paper mill effluent contaminated by many substances including 50.3 ± 1 mg L⁻¹ PCP. The bacteria were incubated in flasks with: contaminated effluent samples, 1.0% glucose, 0.5% peptone and 1 ml of bacterial culture (individual and mixed). The reduction of PCP effects from pulp paper mill effluent by the bacterial strains was remarkable, in respects to their synergic action. *S. marcescens* ITRC S₇ and mixed cultures degraded 85% and 100%. The use of effluent samples was to confirm the ability of bacteria strains to degrade PCP, and then evaluate the

intermediate metabolites. The substances in the flasks consisted of: TCHQ, 2-CP, 6-chlorohydroxyquinol (CHYQ) and 2, 4, 6-TCP. Shah and Thakur (2002) isolated three different strains capable of degrading PCP. They were identified as *Serratia marcescens* (TE1, TE2 and TE4). Degradation potential at 100 mg L⁻¹ PCP was investigated in terms of growth, ring cleavage, chloride release and PCP utilization. Three strains of *S. marcescens* TE1, TE2 and TE4, after 96 h, were able to utilize 52%, 59% and 68%. During this utilization of carbon a source amount of chloride had accumulated in the culture broth. The degradation of PCP by bacterial strains conducted through an oxidative process as indicated by accumulation of degraded products such as CHQ, DCHQ and TCHQ. All intermediary metabolites were used by the bacterial strains. In fact after 96 h *S. marcescens* TE1 was able to utilize 63% of CHQ. However, the amount of PCP utilized by this strain was 42%. *S. marcescens* TE2 was able to remove 62% of DCHQ and 43% of PCP. *S. marcescens* TE4 had a greater capability to degrade all of the compounds, but the maximum utilization of PCP was 68%. *S. marcescens* T4, T1 and T2 were able to degrade 50% and, 65% of TCHQ.

2.4.9. The genus *Sphingomonas*

Sphingomonas genus was separated from *Pseudomonas* by Yabuuchi et al. (1990) and it is often used in a number of bioremediation experiments especially that of CPs (McAllister et al. 1996; Field and Sierra-Alvarez 2008). *Sphingomonas* sp. in some cases showed a rapid mineralization of PCP thanks to the remarkable ability to break down hydrocarbon bonds. Dai and Copley (2004) used the Gram-negative *Sphingobium chlorophenolicum* ATCC 39723, obtained from the American Type Culture Collection, to improve the degradation of PCP using the genome shuffling method. PCP final concentrations that were used in the experiment were: 0.3 and 3 mM PCP. They obtained several strains of *S. chlorophenolicum* and all microorganisms were able to degrade and tolerate PCP much better than that of the wild type. They tested PCP

degradation of the strains in different conditions of contaminant exposure and when bacteria cells were pre-exposed to 50 μM of PCP, both strains, including the wild type, ended up degrading PCP at a higher rate after genome shuffling. The Mutant and wild type strains at a concentration of 0.3 mM PCP, both did a very good job of degrading PCP, although when they added 3mM PCP to the medium only the mutant strain had the ability to grow and degrade PCP. During the experiments tetrachloro-1,4-benzoquinone (TeCBQ) was found. McCarthy et al. (1997a) isolated from highly PCP contaminated soil, a strain of *Sphingomonas chlorophenolica* RA-2. The isolate degraded 100% of PCP at 250 and 300 mg L^{-1} PCP. The principal products of degradation were TCHQ, trichlorohydroquinone (TriCHQ) and 2,6-DCHQ. The final pathway has yet to be defined but it is known that this bacteria can mineralized PCP to CO_2 , H_2O , and Cl^- (McCarthy et al., 1997b). *Sphingomonas* sp. UG30 was used in PCP mineralization and degradation by Alber et al. (2000). In an vitro experiment the authors analyzed the mineralization capacity at 30 mg L^{-1} PCP, adding ammonium phosphate or ammonium nitrate as a nitrogen source. The Optimum PCP degradation of about 65% occurred using ammonium phosphate. Rutgers et al. (1997) showed that PCP affects the growth rate of *Spingomonas* sp. P5 This bacterium uses PCP uniquely as the source of carbon and energy. This experiment was conducted in a continuous liquid continuous culture with on-line measurement and control of the substrate concentration. A Specific growth rate, showed a maximum value of $0.142 \pm 0.004 \text{ h}^{-1}$ at a set-point of PCP concentrations between 37 and 168 μM . At PCP concentrations above 168 μM , the growth rate decreased by inhibition. Further studies of the degradation of PCP with *Spingomonas chlorophenicum* ATCC 39723 were conducted by Huang et al. (2008). The wild type and mutant strains (PcpF) were tested in PCP remediation. In the PcpF strain, they added an orf19 gene which was able to produce great quantities of Glutathionyl-Hydroquinone Lyase, enzyme which is very useful in PCP degradation. The *S. chlorophenicum* ATCC 39723 wild type and PcpF

were cultured in the mineral medium with as well as without glutamate induced with 100 μM PCP and incubated until PCP mineralization. The wild type and PcpF completely degraded 100 μM PCP within 40 min in the presence of glutamate. Contrarily, bacteria grew without glutamate, wild type cells degraded 100 μM PCP in 1 h while, the PcpF only needed 4 h to complete the degradation of PCP. The PcpF strain was more sensitive to PCP toxicity and had a significantly decreased PCP degradation rate, due to the accumulation of the GS-hydroquinone. Thus, PcpF played an important role in PCP degradation and converted the GS-hydroquinone conjugates back to the intermediates of PCP degradation pathways. In addition to *Pseudomonas* sp., Männistö et al. (1999) isolated three other strains that could degrade PCP from *Sphingomonas* sp. (isolates K6, K101, and K74). The PCP degradation capacity was evaluated when the compound was alone or in a mixed solution that contained 80% 2, 3, 4, 6-TeCP and 20% PCP. When PCP was present in mixed solutions with 2, 3, 4, 6-TeCP, it completely was degraded by strain *Sphingomonas* sp. K101. Contritely the strain *spingomonas* sp. K74 partially degraded to about 60%. When PCP was alone at 2 mg L^{-1} PCP, only strain *Sphingomonas* sp. K6 degraded completely PCP in less than 30 days. This indicates that the degradation of PCP in *Sphingomonas* sp. K101 and K74 may have been induced by 2, 3, 4, 6-TeCP. On the other hand, isolate K6 degraded PCP by itself, but not in the mixture solution with 2, 3, 4, 6-TeCP.

Spinghomonas chlorophenolica, notoriously has been able to degrade and dechlorinate PCP which was used by Yang et al. 2005 in batch reactor experiments. The authors showed how a PCP pre exposition of *S. chlorophenolica* increased the ability to degrade the contaminant. In fact when the initial PCP concentration was at 380 mg L^{-1} , the *S. chlorophenolica* completely degraded the PCP within 45.6 h, increasing the PCP concentration from 560 to 720 mg L^{-1} , efficiently decreased PCP to 34.7% and 58.9% during 165 h of incubation. On the other hand, without a pre-exposition between the organism and PCP, the contaminant was removed completely within 89.2 h at 250 mg

L^{-1} PCP. However, the removal efficiency rose to 89%, after 110.8 h, at 400 mg L^{-1} . If the initial PCP concentration was increased above 600 mg L^{-1} , *S. chlorophenolica* could not degraded PCP. Strains of *Sphingomonas* sp. UG30 were used by Alber et al. (2000) in soil perfusion bioreactors in PCP degradation tests. The authors analyzed the PCP mineralization capacity of the bacterium in a glucose medium with ammonium phosphate or ammonium nitrate as a nitrogen source. In this experiment, bioreactors were used at three different PCP concentrations 100, 225 and $500 \text{ mg PCP Kg}^{-1}$ soil. The first two concentrations obtained degradation of 80% and 99%. At $500 \text{ mg PCP Kg}^{-1}$ soil there was no degradation. Another test was conducted by Yang and Lee (2008) using a pure PCP-degrading bacterium strain, identified as *Sphingomonas chlorophenolica* PCP-1, isolated from PCP-contaminated soils in Taiwan. This bacterium was tested in a batch reactors with contaminated water by 160 mg L^{-1} PCP. Depletion of the PCP and the chloride release were measured at different bacterial biomasses (0.14, 0.28, 0.42 and 0.54 g L^{-1}). The results indicated that at 160 mg L^{-1} PCP was completely degraded within 25 h under different bacterial biomass' (dry weight) in the groundwater. It was evident as they increased the biomass of the bacteria, caused a decrease in the degradation of PCP and ca. 110 mg L^{-1} chloride was released by each bacterial concentration within the same period of time.

Alber et al. (2000) used *Sphingomonas* sp. UG30 in PCP mineralization and degradation in statically incubated soil. *Sphingomonas* sp. UG30 was tested at three different PCP concentrations (100, 225 and $500 \text{ mg PCP Kg}^{-1}$ soil) during 22 days of incubation. For the first two concentrations the results showed a degradation of 25% and 65%. At $500 \text{ mg PCP Kg}^{-1}$ soil there was no degradation. *Sphingomonas chlorophenolica* RA2 was used by Colores and Schmidt (2005) in a recovery treatment of laboratory contaminated soil with the following concentrations: 0, 10, 50, 100, or 300 ppm PCP. *S. chlorophenolica* RA2 degraded only 10% in the soil contaminated with 10 ppm PCP, reaching 30% after three weeks of incubation. No degradation was

noted at any other concentrations. Miethling and Karlson (1996) studied PCP mineralization in a sample of soil from Denmark with levels of 30 and 100 mg PCP Kg⁻¹ soil after inoculation with *S. chlorophenolica* RA2. They compared the result with the activity of the same soil without inoculation, determining its natural capacity for PCP mineralization. None of the inoculated soils completely mineralized 30 mg PCP Kg⁻¹ soil within 7 months but showed little to no degradation activity at 100 mg Kg⁻¹ in the same time period (less than 2%). In soil inoculated with 30 mg PCP Kg⁻¹ soil, *S. chlorophenolica* RA2 reduced the mineralization time drastically to only 1 month. At 100 mg Kg⁻¹, mineralization was slower because of the high PCP toxicity but approached completion within 7 and a half months. The inhibition could have been overcome by addition of sawdust (1 g Kg⁻¹ soil), which was shown to increase the mineralization rate.

2.4.10. Other genera

Other bacteria showed a good tolerance and ability to degrade PCP, even at low initial concentrations. They were rarely studied in the presence of PCP, although it would be useful to consider them for future research.

Verma and Singh (2013) have isolated a bacteria identified as *Brevibacterium casei* (TVS-3) able to degrade 1000 mg L⁻¹ PCP concentration. The bacterium degraded 72% PCP within 168 h at pH 7.5 and 35 °C temperature. After 168 h *B. casei* showed maximum PCP utilization of 720 mg L⁻¹ and released 900 mg L⁻¹ chloride ions. Finally *B. casei* to carried out the maximum depletion of PCP, about 82%, at pH 8.0 and 35 °C within 168 h. The predominant Gram-negative bacterial strain, identified as *Agrobacterium radiobacter*, was used by Yu and Ward (1996) in PCP degradation tests, individually and in a combination with *Pseudomonas* sp. After 4 days of incubation at 100 ppm PCP, the capacity of PCP degraded by individual isolates was

lower than observed when the strains were combined. In fact, *A. radiobacter* and the mixed culture degraded 60% and 80%, respectively. Finally, the mass spectrum analysis showed that a principal metabolite of PCP degradation produced by *Pseudomonas* sp. and *A. radiobacter* was tetrachlorophenol (TeCP). From mushroom compost, Webb et al. (2001) isolated a strain known as *Saccharomonospora viridis* which was tested to degrade at concentrations of 10 mg L⁻¹ PCP. The experiment was carried out after 10 days of incubation but within only eight days all of the PCP was degraded. The authors highlighted that *S. viridis* does not possess the ability to degrade PCP but rather transform it into other compost. They proposed this initial pathway for PCP transformation: PCP → TCHQ → TeCBQ. When the PCP concentration was above 20 mg L⁻¹ it resulted in being too toxic for *S. viridis*. Adrian et. al (2007) used PCP as electron acceptors using *Dehalococcoides* sp. (strains 195 and CBDB1) demonstrating that this bacterium could produce a reductive dechlorination of the compounds. Only strains *Dehalococcoides* sp. CBDB1 dechlorinated PCP completely and quite rapidly, within one week of incubation. PCP dechlorination produced a mixture of 3,5-DCP, 3,4-DCP, 2,4-DCP, 3-CP and 4-CP, indicating that several degradation pathways were catalyzed. Männistö et al. (1999) isolated and tested the degradation capabilities of PCP with strains *Nocardioides* sp. (isolates K44 and K103) and *Candidatus comitans* K112. In these experiments, Männistö et al. evaluated the PCP degradation capacity when the compound was independent or mixed in a solution which contained 80% 2, 3, 4, 6-TeCP and about 20% PCP. The mixed solution of 2, 3, 4, 6-TeCP, PCP ended up completely degraded by strains. When PCP was independent at 2 mg L⁻¹, the strain *C. comitans* K112 degraded the PCP completely within the 28 days of incubation. *Nocardioides* sp. K44 and K103 did not degrade the PCP when alone, although they did degraded it completely when mixed with 2, 3, 4, 6-TeCP. This indicates that the degradation of PCP may have been induced by 2, 3, 4, 6-TeCP. *Novosphingobium* sp. MT1 is a bacteria which was isolated in contaminated water and

sand in Finland, presenting a mixture of CP contaminant (Tirola et al. 2002). The substrate was spiked four times with a CPs mixture containing 2,4,6-TCP, 2,3,4,6-TeCP, and PCP, which was the approximate ratios as in the influent groundwater where the bacterium was isolated. *Novosphingobium* sp. MT1 strain showed a good PCP degradation capacity. It completely degraded the PCP after the first spiking (about 150 h) which continued to have a very high level of degradation even after the other three spikings.

Anaerobic bacteria *Actinomycetes* sp., *Streptacidiphilus* sp., aerobic *Rhodococcus erythropolis*, *Amycolatopsis* sp. and *Gordonia* sp. were found to be tolerant and able to degrade PCP in contaminated effluent in a biocathode by Huang et al. (2012). These bacteria, in a mixed culture, were tested at different initial PCP concentrations (5, 10, 20, 30 and 40 mg L⁻¹) with the variable of time being 100 h of incubation. Under PCP concentrations of 20 mg L⁻¹, the PCP was completely degraded within the 100 h. While at 30 and 40 mg L⁻¹ only 15% and 50% respectively. The maximum PCP degradation rate in the cathode was 0.263 ± 0.05 mg/L-h (51.5 mg/g VSS-h) with 60.6% reduction of PCP from 31.2 ± 2.1 mg L⁻¹ to 12.3 ± 2.1 mg L⁻¹ after 3 days. The abiotic control showed a PCP loss of 10.6%, due to the chemical reduction, adsorption, measurement errors and diffusion through the membrane into the anode chamber. Chloride accumulated in the solution was in proportion to the PCP removed, demonstrating microbial dechlorination. At an initial PCP concentration of 30 mg L⁻¹, chloride ions were produced after 72 h, while there was less Cl⁻ released at 40 mg L⁻¹ PCP. Probably due to the inhibition of microorganisms at this concentration. At a high temperature of 50 °C and pH level of 6 the PCP degradation improved. Principal PCP degradation product obtained by experiments were: TCHQ, TriCHQ and 2,6- DCHQ. The specific role of the individual microorganism was not analyzed but in the complex they were able to mineralize PCP in the biocathode.

2.4.11. Bacteria general discussion

The PCP biodegradability in microbiological culture media has been considered in many works, being the initial step for whatever bioremediation work. The majority of these studies are focused on metabolism of single organisms, mixed cultures and degradation pathways under different conditions (pollutant concentration, temperature, pH level and moisture). The Gram-negative *Brevibacterium casei* (Verma and Singh 2012) was the most resistant bacterium able to degrade very high PCP concentration. This bacterium may be potentially useful in PCP bioremediation processes, but despite that, in bibliography there is an only one study in PCP depletion. It might be interesting to increase the knowledge on the interaction between this bacterium and PCP. *Pseudomonas* sp. and *Sphingomonas* sp. are certainly the most tolerant of PCP in respects to bacteria which degrades this compound in the many studies on bioremediation. These two genera have showed excellent capacity also in other pollutants degradation and several of the new genetic studies are focused on them. They are able to remove very high initial concentrations of PCP. A large number of species belonging to *Pseudomonas* sp. has been widely used. *Pseudomonas fluorescens*, versatile bacteria with biocontrol properties, has really paved the way for considerable possibilities in bioremediation strategy. To protect the roots of some plant species against parasitic fungi and bacteria (Haas and Défago 2005). Specific considerations must be thought for *Pseudomonas aeruginosa*. In fact, this bacterium, despite the ability to degrade PCP (although slowly), has some negative attributes like species that can cause disease in animals (including humans) and in plants (He et al. 2004). Decidedly the most innocuous genus listed is *Pseudomonas* (e.g. *P. mendocina*, *P. stutzeri* and *P. veronii*). Essentially for the second genus, *Sphingomonas chlorophenolica* RA-2 is the species that appears in most of the works. *Pseudomonas* sp. and *Sphingomonas* sp. are able to mineralize PCP and the intermediate products of the PCP biodegradation are: TCHQ (Shah and Thakur 2002; McCarthy et al. 1997a),

TCC (Nam et al. 2003), CPs (Kao et al. 2005) and CHQs (Shah and Thakur 2002; McCarthy et al. 1997a) (Table 3). This bacteria has shown excellent results which have been highlighted *Bacillus* sp. Which has the ability to degrade PCP very quickly, either individually or when used in mixed cultures with other *Bacillus* or with microorganisms of other genera. However, a very important point to remember is that in the *Bacillus* genus, there is *Bacillus cereus* which is responsible for a minority of food-borne illnesses, causing severe nausea, vomiting and diarrhea (Kotiranta et al. 2000). Others strains of genus which are reported in this review appear to be extremely weak parasites. The intermediate products of the PCP biodegradation for *Bacillus* sp. are TCHQ (Singh et al. 2009). *Serratia marcescens* was often isolated from many sites highly contaminated with PCP showing a formidable ability in the degradation of PCP caused by a reductive dechlorination. The intermediate products of the PCP biodegradation for *Serratia* sp. are TCHQ and CHQs (Shah and Thakur 2002 Singh et al. 2008; 2009). If it is a naturally occurring reaction, it has potential to be applied to the treatments of PCP contaminated sites e.g. pulp, paper and mill effluent. Although a lower possible use in the bioaugmentation processes because it is a human pathogen, which has been linked to infections in the urinary tract and skin (Hejazi and Falkiner 1997). When used in a mixed culture (e.g. *Bacillus* sp.) showed a good synergistic effect, which increased the percentage of the PCP degraded. *Arthobacter* sp. also showed a high level of tolerance and degradation even though the best performance of PCP removal was only in mixed cultures. The genus *Nocardioides* was not able to degrade PCP when it was alone, but only in the presence of another pollutant despite low initial concentration. *Candidatus comitans* and *Escherichia coli* showed a slow capacity in degrading PCP opposed to *Acinetobacter* sp., *Dehalococcoides* sp., *Kokuria* sp. and *Novosphingobium* sp. which showed a higher capacity of tolerance and degradation to PCP, as well as being quicker than most other strains.

Saccharomonospora viridis degraded at a low PCP concentration, and was not able to mineralize PCP values of 20 mg L⁻¹ are already toxic to these species.

In engineered systems there were few studies available on the biological treatment of PCP. This system in a short time could result an interesting practical system for bioremediation of PCP-contaminated water and soil samples (Table 3); although in some cases they can result very expensive. *Sphingomonas chlorophenolica* was the best microorganism in dealing with PCP degradation in the batch reactor tests. It was able to remove very high concentrations of PCP very quickly. The threshold of tolerance to this pollutant appears to be around 500 mg Kg⁻¹ without pre exposition to PCP. At this value the degradation capacity seem to be inefficient. Moreover, with a PCP pre-contact, *S. chlorophenolica* can tolerate and degrade concentrations around of 750 mg Kg⁻¹ (Yang et al. 2005; Lanthier et al. 2005). *Arthrobacter* sp. showed a high capacity in regards to PCP degradation but it was rather slow (Edgehill 1996). *Desulfitobacterium* sp., which is one of the most versatile strains, could possibly be on the best candidates (having analyzed both the species) for developing the bioremediation processes (Tartakovsky et al. 1999). This bacterium produced TCP as intermediate products of PCP biodegradation. Mixed cultures generally mineralized concentrations of PCP at values of no more than 20 mg L⁻¹. This ability to completely mineralize the PCP and its metabolites, depends essentially on the type of microbial consortium and their synergistic effects. In presence of amendments such as N and P, the ability to degrade PCP, the bacteria strains greatly increased (e.g. *S. chlorophenolica* and *Pseudomonas* sp.).

Physical, chemical, and biological studies on PCP biodegradation in contaminated soil, effluent and sludge's represent an fundamental topic on which much research has been focused (Table 3). Various bacteria have been employed to remove PCP, and obtain complete mineralization. Inoculation with PCP degraders may, in some cases, be the

only way for microbial cleanup of contaminated sites. However, the success of PCP bioremediation is affected by several factors, including the species of microorganisms and site properties (soil characteristics, environmental conditions, pollutants amount and etc). When contaminants such as PCP enter into the soil or water they are subjected to a high number of bio-chemicals processes (Castillo and Bárcenas 1998; McAllister 1996; Field and Sierra-Alvarez 2008). A contaminant may be lost at different rates and to different phases (Stokes et al., 2006). The Remaining fraction present in the soil is not completely available to all organisms, because they can be sequestered by organic and inorganic compounds. Fluvisol, Chernozem, and Arenosol studied by Vítková et al. (2011) which showed a different ability in PCP bioremediation. It was widely noted that a good success of decontamination and detoxification depends mainly on the amount of organic soil matter and other parameters as seen also by Scelza et al. (2008). The species belonging to genus *Spingomonas* and *Pseudomonas* showed an excellent ability to metabolize PCP also at very high initial concentrations; even at 500 mg PCP Kg⁻¹ soil was inhibited. Exactly as in microbiological liquid media, the genus *Pseudomonas* biodegrade PCP in TCHQ. PCP mineralization can in many cases be increased and accelerated (also in less time), above all when there are in the optimal conditions for growth. *Mycobacterium chlorophenolicum* and *Kokuria* sp. showed a high level of tolerance and degradation to PCP. Finally, *Flavobacterium* sp. had the ability to degrade and mineralize high PCP concentrations but this capacity was dependent on the type of soil in which it was tested and the presence of other microorganisms. In fact this genus in the interaction with other microorganisms often tends to have a secondary role.

Table 3. Degradation of PCP by bacteria

Species	Conditions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
<i>Sphingomonas chlorophenolica</i> RA2	Soil	30 - 100 mg Kg ⁻¹	NR	NR	+	Chloride ions	Mietling and Karlson 1996
<i>Mycobacterium chlorophenolicum</i> PCP1	Soil	30 - 100 mg Kg ⁻¹	NR	NR	+	Chloride ions	
<i>Arthrobacter</i> sp. ATCC 33790	Liquid	145 mg L ⁻¹	NR	80 - 100%	NR	NR	Edgehill 1996
<i>Flavobacterium gleum</i>	Liquid	100 ppm	25%	NR	NR	TeCP	Yu and Ward 1996
<i>Pseudomonas</i> sp.	Liquid	100 ppm	20%	NR	NR	TeCP	
<i>Agrobacterium radiobacter</i>	Liquid	100 ppm	60%	NR	NR	TeCP	
Mixed culture	Liquid	100 ppm	80%	NR	NR	TeCP	
<i>Sphingomonas</i> sp. P5	Liquid	37 - 168 µM	+	NR	+	Chloride ions	Rutgers et al. 1997
<i>Sphingomonas chlorophenolica</i> RA-2	Liquid	250 - 300 mg L ⁻¹	100%	NR	+	TCHQ ; TriCHQ ; 2,6-DCHQ	McCarthy et al. 1997
<i>Pseudomonas</i> sp. SR3	Soil	175 ppm	50 - 65%	NR	+	Chloride ions	Plender et al. 1997
<i>Flavobacterium</i> sp. ATCC53874	Soil	125 ppm	50 - 65%	NR	+	Chloride ions	
<i>Pseudomonas</i> sp. Bu34	Liquid	1000 - 4000 mg L ⁻¹	75 - 90%	NR	+	NR	Lee et al. 1998
<i>Mycobacterium chlorophenolicum</i> PCP1	Soil	22 mg Kg ⁻¹	5 - 50%	NR	NR	Chloride ions	Combrisson and Monrozier 1999
<i>Sphingomonas</i> sp. K6	Liquid	0.2 - 2 mg L ⁻¹	100%	NR	+	Chloride ions	Männistö et al. 1999
<i>Sphingomonas</i> sp. K101	Liquid	0.2 - 2 mg L ⁻¹	100%	NR	+	Chloride ions	
<i>Sphingomonas</i> sp. K74	Liquid	0.2 - 2 mg L ⁻¹	60%	NR	+	Chloride ions	
<i>Nocardioides</i> sp. K44	Liquid	0.2 - 2 mg L ⁻¹	+	NR	+	Chloride ions	
<i>Nocardioides</i> sp. K103	Liquid	0.2 - 2 mg L ⁻¹	+	NR	+	Chloride ions	
<i>Candidatus comitans</i> K112	Liquid	0.2 - 2 mg L ⁻¹	100%	NR	+	Chloride ions	
<i>Pseudomonas amygdali</i> K104	Liquid	0.2 - 2 mg L ⁻¹	60%	NR	+	Chloride ions	
<i>Pseudomonas</i> sp.	Liquid	0.405 - 0.474 mg L ⁻¹	90 - 100%	NR	NR	NR	Schmidt et al. 1999
<i>Desulfobacterium frappieri</i> PCP-1	Liquid	1 - 100 mg L ⁻¹	NR	99 - 100%	NR	TCP ; TCP ; PH	Tartakovsky et al. 1999
<i>Arthrobacter</i> sp. ATCC 33790	Liquid	50 - 120 mg L ⁻¹	+	NR	+	Chloride ions	Hamid Mollah and Grant Allen, 1999
<i>Sphingomonas</i> sp. UG30	Liquid	30 mg L ⁻¹	65%	NR	NR	Chloride ions	Alber et al. 2000
<i>Sphingomonas</i> sp. UG31	Soil	100 - 500 mg Kg ⁻¹	+	NR	NR	Chloride ions	
<i>Sphingomonas</i> sp. UG32	Liquid	100 - 500 mg Kg ⁻¹	+	NR	NR	Chloride ions	
<i>Saccharomonospora viridis</i>	Liquid	10 mg L ⁻¹	100%	NR	NR	Chloride ions	Webb et al. 2001
<i>Novosphingobium</i> sp. MT1	Liquid	5% PCP of a mixture	100%	NR	NR	TCHQ ; TeCBQ	Tirola et al. 2002
<i>Serratia marcescens</i> TE1	Liquid	100 mg L ⁻¹	52%	NR	NR	CHQ ; DCHQ ; TCHQ	Shah and Thakur, 2002
<i>Serratia marcescens</i> TE2	Liquid	100 mg L ⁻¹	59%	NR	NR	CHQ ; DCHQ ; TCHQ	
<i>Pseudomonas fluorescens</i> TE3	Liquid	100 mg L ⁻¹	+	NR	72%	CHQ ; DCHQ ; TCHQ	
<i>Serratia marcescens</i> TE4	Liquid	100 mg L ⁻¹	68%	NR	NR	CHQ ; DCHQ ; TCHQ	
<i>Pseudomonas</i> sp. IST103	Liquid	0.1 g L ⁻¹	+	70%	NR	NR	Gautam et al. 2003
<i>Pseudomonas</i> sp. IST103	Soil	0 - 1000 mg L ⁻¹	+	NR	NR	TCHQ	

Table 3. Continued

Species	Conditions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
<i>Sphingobium chlorophenolicum</i> ATCC 39723	Liquid	0.3 / 3 mM	80%	NR	NR	tetrachlorobenzquinone	Dai and Copley 2004
<i>Pseudomonas gladioli</i> M-2196	Liquid	3 μ l L ⁻¹	+	NR	NR	TCC	Nakamura et al. 2004
<i>Pseudomonas gladioli</i> M-2196	Soil	2.5 μ l L ⁻¹	10%	NR	NR	TCC	
<i>Desulfobacterium hafnense</i>	Liquid	0.0013 - 0.364 g L ⁻¹	60%	NR	NR	TCP	Lanthier et al. 2005
<i>Sphingomonas chlorophenolica</i>	Liquid	100 - 800 mg L ⁻¹	+	+	NR	NR	Yang et al. 2005
<i>Sphingomonas chlorophenolica</i> RA2	Soil	0 - 300 ppm	+	NR	+	NR	Colores and Schmidt 2005
<i>Pseudomonas mendocina</i> NSYSU	Liquid	20 - 320 mg L ⁻¹	+	NR	NR	2, 4, 6-TCP ; 2, 4-DCP ; 4-CP ; 2-CP	Kao et al. 2005
<i>Bacillus cereus</i> ITRC S ₆	Liquid	300 mg L ⁻¹	62.75%	NR	NR	NR	Chandra et al. 2006
<i>Serratia marcescens</i> ITRC S ₉	Liquid	300 mg L ⁻¹	86.6%	NR	NR	NR	
<i>Dehalococcoides</i> sp. CBDB1	Liquid	20 μ M	NR	100%	NR	3,5-DCP ; 3,4-DCP ; 2,4-DCP ; 3-CP	Adrian et al. 2007
<i>Arthrobacter</i> sp. ATCC33790	Soil	95.43 - 521 mg Kg ⁻¹	+	NR	NR	NR	Pu and Cutright 2007
<i>Flavobacterium</i> sp. ATCC 21918	Soil	263 - 539 mg Kg ⁻¹	+	NR	NR	NR	
Mixed culture (ATCC33790 + ATCC 21918)	Soil	262 - 539 mg Kg ⁻¹	+	NR	NR	NR	
<i>Serratia marcescens</i> ITRC S ₇	Liquid	300 mg L ⁻¹	90.33%	+	NR	TCHQ ; CHYQ	Singh et al. 2007
<i>Escherichia coli</i> PCP1	Liquid	100 mg L ⁻¹	60%	NR	NR	TCHQ ; DCBQ ; 2,3,4,6-TeCP	Sharma and Thakur 2008
<i>Pseudomonas aeruginosa</i> PCP2	Liquid	100 mg L ⁻¹	15 - 65%	NR	NR	TCHQ ; DCBQ ; 2,3,4,6-TeCP	
<i>Acinetobacter</i> sp. PCP3	Liquid	100 mg L ⁻¹	80%	NR	NR	TCHQ ; DCBQ ; 2,3,4,6-TeCP	
<i>Pseudomonas fluorescens</i>	Liquid	200 μ M	+	NR	NR	PH	Lin et al. 2008
<i>Bacillus</i> sp. ITRC S ₈	Liquid	50.31 mg L ⁻¹	+	+	NR	2-CP ; TCHQ	Singh et al. 2008
<i>Serratia marcescens</i> ITRC S ₉	Liquid	50.31 mg L ⁻¹	+	+	NR	2-CP ; TCHQ	
Mixed culture (ITRC S ₈ + ITRC S ₉)	Liquid	50.31 mg L ⁻¹	94%	+	NR	2-CP ; TCHQ	
<i>Sphingobium chlorophenolicum</i> ATCC 39723	Liquid	100 μ M	100%	NR	+	TCHQ ; TrCHQ ; 2,6- DCHQ	Huang et al. 2008
<i>Pseudomonas testosteroni</i> CCM 7350	Soil	10 - 100 mg L ⁻¹	+	NR	NR	NR	Sejčková et al. 2009
<i>Sphingomonas chlorophenolica</i> PCP-1	Liquid	160 mg L ⁻¹	100%	NR	NR	Chloride ions	Yang and Lee 2008
<i>Bacillus cereus</i> ITRC S ₆	Liquid	50.3 mg L ⁻¹	90 - 100%	NR	NR	TCHQ ; CHYQ ; 2, 4, 6-TCP	Chandra et al. 2009
<i>Serratia marcescens</i> ITRC S ₇	Liquid	50.3 mg L ⁻¹	85 - 100%	NR	NR	TCHQ ; CHYQ ; 2, 4, 6-TCP	
Mixed culture (ITRC S ₆ + ITRC S ₇)	Liquid	50.3 mg L ⁻¹	+	NR	NR	TCHQ ; CHYQ ; 2, 4, 6-TCP	
<i>Bacillus cereus</i> (DQ002384)	Liquid	300 mg L ⁻¹	62.75%	+	NR	TCHQ ; CHYQ ; 2,3,4,6-TeCP	Singh et al. 2009
<i>Serratia marcescens</i> (AY927692)	Liquid	300 mg L ⁻¹	85.5%	+	NR	TCHQ ; CHYQ ; 2,3,4,6-TeCP	
<i>Serratia marcescens</i> (DQ002385)	Liquid	300 mg L ⁻¹	90.33%	+	NR	TCHQ ; CHYQ ; 2,3,4,6-TeCP	
Mixed culture (DQ002384 + AY927692 + DQ002385)	Liquid	300 mg L ⁻¹	93%	+	NR	TCHQ ; CHYQ ; 2,3,4,6-TeCP	
<i>Acinetobacter</i> sp. ISTPCP-3	Liquid	20 - 250 mg L ⁻¹	+	NR	NR	2,3,5,6-TCHQ ; DCBE	Sharma et al. 2009
<i>Pseudomonas stutzeri</i> CL7	Liquid	50 - 600 mg L ⁻¹	90 - 95%	NR	+	Chloride ions	Karn et al. 2010a
<i>Bacillus megaterium</i> CL3	Liquid	50 - 600 mg L ⁻¹	80 - 100%	+	NR	Chloride ions	Karn et al. 2010b

Table 3. Continued

Species	Condictions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
Mixed culture (CL3 + CL5 + CL11)	Liquid	100 mg L ⁻¹	80 - 100%	+	NR	Chloride ions	Karn et al. 2011
<i>Kokuria</i> sp. CL2	Liquid	100 mg L ⁻¹	100%	+	NR	Chloride ions	
<i>Bacillus</i> sp.	Liquid	500 µg ml ⁻¹	56%	NR	NR	Chloride ions	
<i>Pseudomonas testosteroni</i> CCM 7350	Soil	10 - 100 mg L ⁻¹	+	NR	NR	NR	Vítková et al. 2011
Mixed culture	Liquid	5 - 40 mg L ⁻¹	15 - 100%	+	+	Chloride ions	Huang et al. 2012
<i>Brevibacterium casei</i> TVS-3	Liquid	1000 mg L ⁻¹	82%	+	NR	Chloride ions	Verma and Singh 2013

NR: Not reported ; +: denotes positive observation ; - denotes no response;

2.5. PCP degradation by fungi

Only during the last forty years PCP fungal bioremediation received some consideration (McAllister et al. 1996; Gadd 2001; Pointing 2001; Singh 2006; Field and Sierra-Alvarez 2008; Rubilar et al. 2008). Currently degradation of PCP by Ascomycetes, Basidiomycetes, yeast, Deuteromycetes and Zygomycetes has been widely studied (McAllister et al. 1996; Gadd 2001; Singh 2006). Ascomycetes showed good results as the disappearance of PCP occurs both in PH-oxidase and oxidase ways. Basidiomycetes deplete PCP moderately, irrespective of high producers of PH-oxidase. Few studies occurs in yeast and other fungi or simil-fungi and PH-oxidase are not produced. Unlike bacteria, fungi do not normally use PCP as source of carbon or energy. PCP degradation is not the consequence of specific enzymes used for these functions. In fact, in fungi, this process occurs through co-metabolic reactions using fungal enzymes, which generally were slotted for other purposes. The biodegradation capacity of some fungi for PCP has shown that they can tolerant very high concentrations such as 500 – 1000 mg L⁻¹ PCP. Even if fungi are not completely efficient in respect to PCP degradation in a liquid culture or soil (McAllister et al. 1996; Gadd 2001). The Fungi groups most commonly used in experiments of PCP degradation are Basidiomycetes agents of White and Brown-rot. Among these, the genus *Phanerochaete*, *Anthracoxyllum* and *Trametes* have received more attention due to their better results, even when a number of other groups of fungi were tested in PCP degradation: Zygomycetes, Ascomycetes and Deuteromycetes (Table 4).

2.5.1. The genus *Anthracoxyllum*

Over the last ten years, white-rot fungi widely used in bioremediation experiments is *Anthracoxyllum discolor*. It is a Chilean fungus from Patagonia which has showed an excellent capacity in bioremediation versus several toxic compounds such as PCP and

Polycyclic aromatic hydrocarbon (PAH) (Diez et al. 2010). In this genus, which is widespread in tropical regions, there are only ten species until now known.

Tortella et al. (2008) evaluated *Anthracophyllum discolor* Sp4 capacity in the biodegradation of some CP compounds which included PCP at 25 mg L⁻¹. After 15 days of incubation, *A. discolor* Sp4 degraded 96% of PCP. PCP degradation by fungi in a liquid medium was correlated only by the ligninolytic enzyme production. Maximum production of manganese peroxidase was detected in *A. discolor* Sp4 between 3 and 6 days of incubation while lignin peroxidase was produced between 6 and 9 days of incubation. Laccase was not detected.

During the last few years *Anthracophyllum discolor* has been widely used in PCP degradation in soil. Rubilar et al. (2011) investigated the bioremediation capacity in Chilean andisol soil contaminated with 250 and 350 mg PCP Kg⁻¹ soil using a strain of *A. discolor*. The fungus strain was used in experiments as free and immobilized in wheat grains (a lignocellulosic material). At initial PCP concentrations of 250 and 350 mg Kg⁻¹ soil, immobilized *A. discolor* removed 80% and 93.2%. In the biotic controlled soil only 50% and 62.6% of PCP was removed at levels of 250 and 350 mg PCP Kg⁻¹ soil. This difference in PCP removal could be due to the synergistic effects occurring between fungi and autochthonous microorganisms. In the sterile soil without fungus (the abiotic control), for both PCP concentrations tested the amount of pollutant removed was 40%, mainly due to the previously mentioned characteristics of the Chilean Andisols which have particularly efficient sorbents for CPs (Cea et al. 2005). Rubilar et al. (2007) carried out a series of laboratory-based studies to determine the range of PCP concentration in soils (100, 250 and 350 mg PCP Kg⁻¹ of soil) which could be degraded in slurry soil flasks by *Anthracophyllum discolor*. The fungus isolate degraded all PCP but only at the initial concentration of 100 mg PCP Kg⁻¹ soil, while for the other two concentrations 250 and 350 mg PCP Kg⁻¹ soil around 50% of the

contaminant was recovered. In *A. discolor*, PCP degradation metabolites were evaluated using GC/MS analysis. The main reaction of PCP degradation was methylation with the production of PCA. The second reaction identified was hydroxylation in the form of TCHQ, which is then methylated to generate tetrachloro-1, 4-dimethoxybenzene, followed by successive dechlorination reactions to form 2, 5-dichloro-1, 4-dimethoxybenzene and 2-chloro-1, 4-dimethoxybenzene. A series of demethoxylation, carboxylation, reduction, and methylation reactions were conducted to form 3, 4-dimethoxybenzaldehyde and then the formation of CO₂ for the complete mineralization. Cea et al. (2010) reported a bioaugmentation essay with *Anthracyllum discolor* in a soil contaminated with PCP and evaluated its impact on the microbial soil community. In this experiment three types of microcosm soils (contaminated with 250 mg PCP Kg⁻¹ soil) were created: fresh soil, fresh soil plus wheat straw and fresh soil plus wheat straw inoculated with *A. discolor*. Manganese peroxidase and laccase activity were higher in the presence of the white-rot fungus while the PCP that was removed after 28 day of incubation was 93.6% for the fresh soil plus wheat straw and about 87% in the fresh soil plus wheat straw inoculated with *A. discolor*.

2.5.2. The genus *Mucor*

Members of the genus *Mucor* were extensively studied for interesting human and plant pathology. It is found commonly in soil, especially on rotten vegetable matter, and has showed discrete ability for direct contaminant degradation such as PCP.

Szewczyk et al. (2003) tested the growth and degradation abilities of fifteen fungal strains isolated from contaminated areas, in the presence of compounds such as PCP. Among these strains there was *Mucor ramosissimus* IM 6203 that in the PCP degradation process released an intermediate product of 2, 3, 5, 6-TCHQ. The PCP

degradation by *M. ramosissimus* IM 6203 was improved significantly in a medium with overworked oil where only 8.3% of PCP and 4.3% of 2,3,5,6-TCHQ were found after 7 days of incubation, starting from 10 mg L⁻¹PCP. Szewczyk and Dlugoski (2009) evaluated PCP degradation and metabolites formed in cultures of *Mucor ramosissimus* IM 6203 with different optimized mediums. After 240 h, 90% of PCP was removed from an initial concentration of 10 mg L⁻¹ and the following metabolites were identified: 2,3,5,6-TCHQ and some anisoles such as pentachloromethoxybenzene. Carvalho et al. 2011 detected the capacity of *Mucor plumbeus* DSM 16513 to remove PCP in a liquid culture and its pathway of PCP degradation using liquid chromatography coupled with quadruple time-of-flight mass spectrometry. In PCP degradation experiments the presence or absence of glucose was a very important variable. When glucose was added to the culture, all PCP was removed during 4 days of incubation. While, after 60 days without glucose only 80% was degraded. The pathway of PCP degradation exhibited the presence of TCHQ, TriCHQ and phase II-conjugated metabolites. Carvalho et al. (2009) studied the co- and direct metabolism of PCP using *Mucor plumbeus* DSM 16513 in experiments of percentage of PCP decay, under co-metabolic conditions with PCP concentration being between 5 and 15 mg L⁻¹. *M. plumbeus* DSM 16513 was able to degrade PCP while at 15 mg L⁻¹ the strains failed biotransformation of PCP. Experiments of PCP decay in fungal cultures under metabolic conditions showed that *M. plumbeus* DSM 16513 removed 85% of PCP up to 5 mg L⁻¹ (maximum value tested).

2.5.3. The genus *Penicillium*

In the genus *Penicillium* there are ubiquitous soil fungi widespread in all world area. It is commonly present wherever organic material is available. During the last thirty years, *Penicillium* spp. have demonstrated remarkable ability to degrade different

xenobiotic compounds and could be potentially interesting for the development of bioremediation processes for pollutant transformation/mineralization.

Carvalho et al. (2009) studied the co- and direct metabolism of PCP by *Penicillium* fungal strains isolated from the cork-colonizing community. The species that were isolated are: *P. glabrum* DSM 16516, *P. olsonii* DSM 16515, *Eupenicillium hirayamae* (anamorph state of *P. hirayamae*), *P. brevicompactum*, *P. glandicola*, *P. variabile*, *P. diversum*, *P. decumbens*, *P. janczewskii*, *P. corylophilum*, *P. adametzii*, *P. fennelliae* and *P. restrictum*. In experiments of mycelium growth and PCP decay (%) under co-metabolic conditions with PCP concentrations being between 5 and 20 mg L⁻¹, all fungi were able to degrade PCP. But the maximum capacity to remove PCP, at 20 mg L⁻¹, was detected only by *P. brevicompactum*, *P. olsonii* DSM 16515 and *P. janczewskii*, with PCP decay of 56% and 59%. Experiments dealing with PCP decay in fungal cultures under metabolic conditions showed that *P. glandicola* and *P. janczewskii* removed PCP until 5 mg L⁻¹ (maximum value tested) for a result of 34%, 67%, and 85%. Only in co-metabolic conditions were produced and identified a PCP metabolic intermediate. CHQ was recovered in the cultures of *P. corylophilum*, *P. glabrum* DSM 16516, *P. glandicola*, *P. janczewskii* and *P. variabile*; 2,6-dichloro-1,4-benzoquinone (DCBQ) in the cultures of *P. decumbens*; and finally TeCBQ in the cultures of *P. adametzii*. Taseli and Gokcay (2005) isolated and studied a *Penicillium camemberti* for its ability to degrade PCP as well as other chlorinated compounds. The batch experiments were conducted in shake flasks using PCP as a co-substrate and *P. camemberti* removed around 56% PCP. Experiments in shake flasks, produced 86% of the PCP removal after 21 days.

2.5.4. The genus *Phanerochaete*

The genus *Phanerochaete* is a widespread group of saprophytic and wood decay fungi. It is a secondary decomposer of both hardwood and softwood. This ability has generated much interest in bioremediation process as an environmentally benign alternative to the chemical bleaching.

It has been shown to detoxify PCP by the methylation process using its lignin-degrading enzymatic tools (McAllister et al. 1996; Gadd 2001; Field and Sierra-Alvarez 2008). This fungus has shown extensive and rapid conversion of PCP in other compounds. Aiken and Logan 1996 studied the degradation of 250 mg L⁻¹ PCP by *Phanerochaete chrysosporium* BMK-F-1767 in a static flask culture using ammonium lignosulphonates (waste product of the papermill industry) as a carbon and nitrogen source. When ammonium lignosulphonates was used as the nitrogen source, PCP removal was 75%. When ammonium lignosulphonates was used as a carbon source, PCP removal was 72%. When *P. chrysosporium* BMK-F-1767 grew on a nitrogen-limited glucose ammonia medium, it removed 95% of PCP. Ryu et al. (2000) investigated the roles and activity of lignin peroxidase, manganese peroxidase and laccase in biodegradation of 30 mg L⁻¹ PCP using *Phanerochaete chrysosporium* IFO 31249. After 15 days *P. chrysosporium* FO 31249 showed low enzymatic activity following a degradation of 72.6 %. Chiu et al. (1998) studied the tolerance, bio-sorption and biodegradation capacity (these last two activities in relation to the fungi biomass dry wet) in the presence of 25 mg L⁻¹ PCP for *Phanerochaete chrysosporium* M1. The tolerance to PCP was very high for *P. chrysosporium* M1 as well as the bio-sorption and biodegradation capacity. Tortella et al. (2008) carried out a study on the ability of *Phanerochaete chrysosporium* CECT-2798 in a biodegradation test with 25 mg L⁻¹ PCP. After 15 days of incubation, *P. chrysosporium* degraded 72% of PCP. Randy and Gold (2000) evaluated intermediate products involved in PCP degradation with *Phanerochaete chrysosporium* OGC101. After 30 hours of incubation, 10% of PCP

was degraded in a nitrogen-limited medium while 90% of PCP was degraded in optimum nutrient conditions. The pathways for the degradation of PCP were elucidated by the characterization of the fungal metabolites and oxidation products generated by purified lignin peroxidase and manganese peroxidase. The Oxidative dechlorination reaction of PCP produced TeCBQ. The quinone was subsequently reduced to tetrachlorodihydroxybenzene, that with another four successive reductive dechlorinations produced 1, 4-hydroquinone and latter formed 1, 2, 4-trihydroxybenzene. Alternatively TeCBQ was converted to 2, 3, 5-trichlorotrihydroxybenzene, which undergoes successive reductive dechlorinations, which produced 1, 2, 4-trihydroxybenzene. Finally, 1, 2, 4-trihydroxybenzene in each of the pathways was ring-cleaved, with subsequent degradation to CO₂.

The genus most frequently used for PCP remediation in soil, sediment and sludge has been *Phanerochaete*. Rubilar et al. (2011) investigated the bioremediation capacity in Chilean andisol soil contaminated with 250 and 350 mg PCP Kg⁻¹ soil using *Phanerochaete chrysosporium* CECT-2798. The fungus strain was incorporated as free and immobilized in wheat grains, a lignocellulosic material. A PCP concentration of 250 and 350 mg PCP Kg⁻¹ soil, *P. chrysosporium* CECT-2798 removed 65% and 79%. In the controlled soil with wheat grains only 50% and 62.6% were removed at 250 and 350 mg PCP Kg⁻¹ soil. In the sterile soil without fungus (the abiotic control), for both PCP concentrations 250 and 350 mg PCP Kg⁻¹ soil the amount of pollutant removed was 40%, probably due to the Chilean Andisols, which are particularly efficient sorbents for CPs, mainly the allophane–ferrihydrite associations with organic matter (Cea et al. 2005). Pfender et al. (1997) used in a laboratory-scale bioremediation in a soil microcosm in a bottle amended with 175 ppm PCP, as well as two bacteria other than just *Phanerochaete sordida*. Over 35% of the available PCP was transformed into PCA after 56 days, while only 10% was mineralized. Okeke et al. (1996) determined the temperature, soil moisture potential and initial pH levels might influence the

transformation of PCP by *Phanerochaete chrysosporium* BKM 1767. This fungal strain showed the highest levels of degradation, about 75% at 25°C and with the soil pH level of 4.0. On the other hand, the extent of PCP degradation related to soil moisture content was higher for *P. chrysosporium* BKM 1767, about 85%. Jiang et al. (2006) investigated the reduction of PCP in contaminated soil inoculating it with free and immobilized *Phanerochaete chrysosporium* BKM-F-1767. Parallel beakers were adopted with the same components of soil, yard waste, straw, bran for aerated composting and 100 mg PCP Kg⁻¹ soil. In the soil inoculated with *P. chrysosporium* BKM-F-1767 (free and immobilized), 90% of the PCP was removed during the 60 days of incubation, while in the same time span the controlled soil which was without inoculation only degraded 50% of PCP. Leštan and Lamar (1996) evaluated the PCP degradation in a soil microcosm of *Phanerochaete chrysosporium* ATCC 42725 and *Phanerochaete sordida* HHB-8922-Sp. After 4 weeks of incubation in a soil artificially contaminated with 100 µg g⁻¹ PCP and inoculated with a 3% pelleted fungal inoculums, both fungi showed a good capacity to convert PCP to PCA. *P. sordida* HHB-8922-Sp removed 92% of the PCP at initial concentrations. PCP methylation was reported for *P. chrysosporium* ATCC 42725 and *P. sordida* HHB-8922-Sp, which transformed PCP to PCA.

2.5.5. The genus *Pleurotus*

Pleurotus is a genus that includes some eaten mushrooms that are found in both tropical and temperate climates throughout the world. It has been used widely in mycoremediation of pollutants such as petroleum, PAH and CPs.

Law et al. (2003) used a spent compost of oyster mushroom *Pleurotus pulmonarius* in PCP degradation and analysis of the processes of biodegradation of a xenobiotic compound. With only 5% of spent compost mushroom of *P. pulmonarius* removed

88.9% (18.8% biosorption and 70.1% biodegradation) of 2 mg L⁻¹ PCP. Further increases in the amount of fungi showed no improvement in the total removal efficiency. For concentrations ranging from 10 to 100 mg L⁻¹ PCP, the trends of *P. pulmonarius* to remove PCP efficiently was between 60% and 80%. Degradation of PCP with *P. pulmonarius* involves dechlorination, methylation, carboxylation and ring cleavage with abundant release especially of TCHQ and TCP. Ryu et al. (2000) investigated the roles and trend of lignin peroxidase, manganese peroxidase and laccase in the biodegradation of 30 mg L⁻¹ PCP using *Pleurotus* sp. KFCC 10943. After 15 days of incubation *Pleurotus* sp. KFCC 10943 showed low enzymatic activity but degraded 70.33% of PCP. Chiu et al. (1998) detected biodegradation capacity in the presence of 25 mg L⁻¹ PCP for *Pleurotus pulmonarius* PL-27. The strain M51 and *P. pulmonarius* PL-27 showed the highest degradative capacity, it being 13 and 10 mg PCP for a gram of mycelium dry wet. Chloroanisols were PCP breakdown intermediates (Table 4). de Souza et al. (2011) investigated PCP removal and adsorption by *Pleurotus pulmonarius* CCB19 in submerged cultures, formed with basal or corn cob medium, in the presence and absence of laccase. When PCP was added at a final concentration of 25 mg L⁻¹, the laccase production considerably increased and 70% of PCP was removed after 96 h. Instead with low laccase activity the removal of PCP was less than 20%. The amount of PCP adsorbed in the mycelial mass was about 10% whether it was obtained in the corn cob medium with laccase or in the basal cultures without laccase. Ramesh and Pattar (2009) tested the biodegradation ability of PCP by *Pleurotus ostreatus*. The fungal strain showed a peak in laccase activity after 30 days of incubation which produced the highest amount of PCP removed. In a static culture *P. ostreatus* degraded 100% of 50ppm PCP during 30 days of incubation.

2.5.6. The genus *Rhizopus*

Rhizopus is a common saprophytic fungi on plants and specialized as animal parasites. A few species of *Rhizopus* are known to cause disease in humans as well as *Rhizopus oryzae*. It is the principal cause of zygomycosis. They are found on a wide variety of organic substrates.

Cortés et al. (2001) that studied PCP degradation in a solid-state culture with a strain of *Rhizopus nigricans*. This fungus displayed high tolerance to growth in the presence of PCP (up to 100 mg L⁻¹) and degraded 60% within 24 h and 100% after 120 h of 12.5 mg L⁻¹PCP. Tomasini et al. (2001) found a strain of *R. nigricans* able to adsorb and degrade PCP in a submerged culture. They found that *R. nigricans* adsorbed PCP and that its adsorption capacity was higher when they increased the PCP concentration in a liquid medium. The biomass of *R. nigricans* adsorbed between 0.004 and 0.15 mg PCP mg mycelium⁻¹. Moreover the fungus completely removed 12.5 mg L⁻¹ of PCP within 6 and 8 days with a mycelium age of 48 and 96 h. León-Santiestebán et al. (2011) described PCP absorption in a nylon fiber in which *Rhizopus oryzae* ENHE was immobilized. Various immobilization techniques were evaluated but, those that produced more biomasses were: cultures with nylon cubes that contained PCP at an equilibrium concentration and nylon at an equilibrium concentration amended with 14 mg PCP g⁻¹ nylon. Two initial PCP concentrations of 12.5 and 25 mg L⁻¹ were tested. In both cultures, PCP removal was similar: after 48h in the cultures with 12.5 mg L⁻¹ PCP 88.6% of contaminate was removed and in cultures with 25mg L⁻¹ PCP, 85.7% was removed. In 72 h for both concentrations the fungus immobilized in nylon absorbed 100% of PCP.

2.5.7. The genus *Trametes*

Members of the genus *Trametes* were extensively studied for interesting activity in medicine and plant pathology. The Aggressive white rot fungi which is world spread, thanks to its enzymes which could be an excellent candidate for direct PCP degradation (McAllister et al. 1996; Gadd 2001; Field and Sierra-Alvarez 2008).

Ullah and Evans (1999) analyzed the ability of *Coriolus versicolor* to deplete PCP comparing inoculated and un-inoculated wheat husk incubated with 200 ppm PCP. In a second experiment they detected PCP degradation by wheat husk inoculated with *C. versicolor* increasing concentrations from 50 to 200 ppm PCP. When wheat husk was inoculated, the PCP was completely removed at any concentration after 72 h. While when the authors used un-inoculated wheat husk only 70% of 200 ppm PCP was depleted. Walter et al. (2003) evaluated nine *Trametes* sp. strains with the potential for bioremediation of 50mg L⁻¹ PCP. The fungi were identified as: *Trametes* sp. HR192, *Trametes* sp. HR196, *Trametes* sp. HR197, *Trametes versicolor* HR131, *Trametes versicolor* HR154, *Trametes versicolor* HR160, *Trametes versicolor* HR275, *Trametes versicolor* HR277 and *Trametes versicolor* HR445. The PCP remaining in the liquid fraction after 42 days of stationary incubation was evaluated and the highest degradation capacity was found to be in *Trametes versicolor* HR275 where 100% of PCP was removed. In correlation to PCP degradation there was also detected the presence and production of laccase. For the genera *T. versicolor* the laccase activity was high and the enzyme production varied with time. Ullah et al. (2000) used a system of different solid substrates to grow *Coriolus versicolor* FPRL-28A. They evaluated laccase activity and the removal of PCP from aqueous effluent. Substrates included wood chips, cereal grain, wheat husk and wheat bran. Higher activity of laccase occurred with wheat husk and wheat bran. Laccase in wheat husk and wheat bran cultures removed 75% – 80% of 50 ppm PCP within 24 h, all the way to 100% after 120 h. in a 5-l stirred tank reactor with wheat pellets uninoculated and inoculated with

C. versicolor FPRL-28A after 30 days of incubation detected the removal capacity of 100 ppm PCP. The inoculated pellet removed 90% PCP during 100 minutes while uninoculated, during the same period of time removed only 50%. González et al. 2010 tested the white-rot fungi *Trametes pubescens* CBS 696.94 in CPs bioremediation and between various compounds used PCP. The experiments were carried out with an initial PCP concentration of 30 mg L⁻¹ and in the absence or presence of supplemented glucose to obtain a final concentration of 1.75 g L⁻¹. After 13 days of incubation there were no differences in PCP degradation with or without glucose and chloride. In both cases 77% of PCP was degraded. Ryu et al. (2000) used lignin peroxidase, manganese peroxidase and laccase by *Trametes* sp. KFCC 10941 in the biodegradation of 30 mg L⁻¹ PCP. After 15 days of incubation *Trametes* sp. KFCC 10941 showed higher enzymatic production, above all with that, of laccase and manganese peroxidase but, on other hand, only 64% of PCP was degraded. Tortella et al. (2008) used *Trametes hirsuta* Ru-008, *Trametes versicolor* Ru-107 and *Trametes versicolor* Ru-0030 in a biodegradation test with PCP at an initial concentration of 25 mg L⁻¹. All other fungi degraded the PCP under 50%, while for three strains, *T. versicolor* (Ru-0030 and Ru-008) and *T. hirsuta* Ru-008, PCP caused an inhibitory effect on growth and enzymatic production. Gaitan et al. (2011) using laccase produced by white-rot fungus *T. pubescens* CBS 696.94 evaluated the PCP degradation capacity in a shake flask. Two laccase iso enzymes with different molecular weights were isolated and identified. After 8 hours of reaction, 41% of 15 mg L⁻¹ PCP was removed in a mixture with other CPs. Ramesh and Pattar (2009) tested in-vitro the biodegradation capacity of PCP with *T. versicolor*. The fungus isolates showed a peak in laccase activity after 30 days of incubation which resulted in the highest amount of PCP removed. In a static culture studies *T. versicolor* degraded 96.14% of PCP within 30 days of incubation. Pallerla and Chambers (1999) investigated the capacity of *T. versicolor* to degrade 25 mg L⁻¹

PCP after 12 h of incubation in continuous polyurethane immobilized fungal fluidized bed bioreactor. *T. versicolor* degraded about 99% of PCP after 12 h.

Excellent results in PCP degradation in soil were shown by *Trametes*. The ability of the Brazilian basidiomycetes to degrade PCP in soils recovered from areas contaminated with organochlorine industrial residues was studied by Machado et al. (2005). *Trametes villosa* CCB176 and *Trametes villosa* CCB213 were tested for tolerance and degradation at high PCP concentrations in soil. Fungi were inoculated into the soil containing 1278 mg PCP Kg⁻¹ soil supplemented with gypsum and sugar, which the authors evaluated for the PCP depletion percentages. *T. villosa* CCB213 reduced 58% the PCP present in the contaminated soil after 90 days of incubation. Both *Trametes* strains mineralized PCP with the successive production of chloride ions during growth, indicating dehalogenation of the molecule and the conversion of PCP to PCA. Walter et al. (2005) used an isolate of *Trametes versicolor* HR131 in field-scale bioremediation of PCP. They devised an engineered soil cell to develop biopiles for fungi bioremediation of aged PCP-contaminated soil from a former timber treatment site. The soil cells were engineered to allow: forced aeration, irrigation, leachate collection, monitoring of temperature and soil humidity. PCP degradation and fungal survival were monitored at regular intervals for 2 and a half years. The PCP field remediation using *T. versicolor* HR131 declined from 1000 mg PCP Kg⁻¹ soil to 100 mg PCP Kg⁻¹ soil within one year. Decreasing at 4 mg PCP Kg⁻¹ soil in two years. At the end of the experiment there was little PCA detected, confirming earlier findings that PCA may not be an intermediate metabolite of PCP transformation by *T. versicolor* HR131. Leštan and Lamar (1996) detected the fate of PCP in soil microcosm inoculated by *Trametes versicolor* MD-277. In the soil artificially contaminated with 100 µg g⁻¹ of PCP and inoculated with 3% pelleted fungal inoculums, *T. versicolor* MD-277 transformed PCP to PCA after 4 weeks degrading 86% of PCP. Tuomela et al. (1999) investigated the fate of PCP in autoclaved soil supplemented with straw and inoculated with the white-rot fungus

Trametes versicolor PRL 572. This strain during 42 days of incubation mineralized about 29% of the PCP and at the end of experiment only trace amounts of PCA and 2,3,4,6-tetrachloroanisole were detected.

2.5.8. The genus *Trichoderma*

Trichoderma is a genus common in soil with interesting capabilities as potential bioremediator for environmental cleanup and as biological control agent versus numerous plant diseases. All these capacity are possible thank to production of extracellular enzymes.

(Tripathi et al. 2013). Carvalho et al. (2009) studied the PCP degradation using *Trichoderma longibrachiatum* DSM 16517 in experiments of mycelium growth and percentage of PCP decay, under co-metabolic conditions with PCP concentration being between 5 and 15 mg L⁻¹. *T. longibrachiatum* DSM 16517 was able to degrade PCP while at 15 mg L⁻¹ the strains failed biotransformation of PCP. Rigot and Matsumura (2002) using *Trichoderma harzianum* 2023 evaluated PCP degradation at 10ppm as an initial concentration. After 9 days of incubation PCP was entirely metabolized, which was quickly and stoichiometrically converted to PCA.

2.5.9. Other genera

Guiraud et al. (2003) studied the bioremediation capability of PCP by *Absidia fusca* detecting the performance of two strains isolated from different environment. After 4 days of incubation the strain1 and strain 2 degraded 41 and 33%, respectively, of 100 mg L⁻¹ PCP. Walter et al. (2003) evaluated a pool of 367 white-rot fungi, native to New Zealand, which are usable in PCP bioremediation. After several tests, some isolates were screened for PCP degradation (50 mg L⁻¹ PCP) in-vitro. The fungi identified were: *Abortiporus biemmis* HR145, *Oudemansiella australis* HR345,

Peniophora sacrata HR226, *Peniophora sacrata* HR235, *Peniophora sacrata* HR240, *Peniophora sacrata* HR241, *Rigidoporus catervatus* HR316 and *Stereum fasciatum* HR348. PCP remaining in the liquid fraction after 42 days of stationary incubation was evaluated and had a high degradation capacity for all strains of *Peniophora sacrata* (100% PCP removed). The correlation to PCP degradation was detected also in the presence and production of laccase. The genera *P. sacrata* laccase activity was high at different points in the experiments. Chiu et al. (1998) detected the tolerance, bio-sorption and biodegradation capacity (these last two activities were in relation to fungi biomass' dry wet) in the presence of 25 mg L⁻¹ PCP for various fungi as *Armillaria gallica* 1039, *Armillaria gallica* 1057, *Armillaria mellea* M51, *Ganoderma lucidum* HK-1, *Lentinula edodes* L54, *Lentinula edodes* L67, *Lentinula edodes* L68, , *Polyporus* sp. Cv-1 and *Volvariella volvacea* V34. The tolerance was higher for *A. gallica* 1039, *A. gallica* 1057 and *A. mellea* M51 while any or all strains tolerated 100 mg L⁻¹ PCP. *Polyporus* sp. Cv-1 possessed the greatest biosorption capacity, about 31 mg PCP for a gram of mycelium dry wet. Chloroanisols were PCP breakdown intermediates for almost all fungi. Tortella et al. (2008) carried out the first report on the ability of several indigenous wood-rotting fungi from Chile to produce hydrolytic and ligninolytic enzymes during the biodegradation of some of the xenobiotic compounds like PCP. Strains were identified and used in laboratory tests on the biodegradation with concentrations of 25 mg L⁻¹ PCP. The Fungi used were: *Lenzites betulina* Ru-30, *Inonotus* sp. Sp2, *Stereum* sp. Ru-24, *Galerina patagònica* Sp3, *Stereum hirsutum* Sp1 and *Stereum hirsutum* Ru-104. After 15 days of incubation *L. betulina* Ru-30 and *G. patagònica* degraded PCP by 80% and 88%. All other fungi degraded the PCP under 50%, while the strains *S. hirsutum* Ru-104 PCP caused an inhibitory effect on the growth and enzymatic production. PCP degradation by fungi in a liquid medium has been correlated with that of ligninolytic enzyme production. In fact, manganese peroxidase was detected in all strains tested. in *L. betulina* Ru-30 and *G. patagònica*

produced a maximum activity of between 3 and 6 days of incubation. Lignin peroxidase was produced in *S. hirsutum* Ru-104, and *G. patagònica* Sp3 between 6 and 9 days of incubation. Laccase was never detected. Scelza et al. (2008) used *Byssoschlamys fulva* in PCP removal experiments in a liquid medium with 12.5 and 25 mg L⁻¹ PCP. The isolates of *B. fulva* degraded 20% of both PCP concentrations during 8 days of incubation. Montiel-González et al. (2009) used the plasmids pVELipA and pTAAMnPI (Stewart et al. 1996), containing lignin peroxidase and manganese peroxidase cDNA, recovered by white rot fungi *Phanerochaete chrysosporium*, for the transformation of *Amylomyces rouxii*. Sixty-nine *A. rouxii* elements were obtained, but only two were chosen for testing PCP removal in a submerged culture because they showed the highest peroxidase activity: CTL4 (lignin-peroxidase) and CTM5 (manganese-peroxidase). CTL4 and CTM5 removed 95% of 12.5 mg L⁻¹ PCP, compared with only 55% of the *A. rouxii* wild type after 120 h of incubation. After 144 h of incubation, two of the elements were able to remove 100% of the initial PCP, whereas the original strain removed only 49%. Carvalho et al. (2009) studied the co- and direct metabolism of PCP using these fungal strains: *Chrysonilia sitophila* DSM 16514, and *Cladosporium herbarum*. In experiments of mycelium growth and percentage of PCP decay, under co-metabolic conditions with PCP concentration being between 5 and 15 mg L⁻¹, both fungi were able to degrade PCP while at 15 mg L⁻¹ the strains failed biotransformation of PCP. Finally, CHQ was recovered in the cultures of *C. sitophila* DSM 16514 and DCBQ in the cultures of *C. herbarum*. Ramesh and Pattar (2009) tested in-vitro the biodegradation capacity of PCP by five selected isolates of white-rot fungi: *Laetiporus cincinnatus*, *Fomes fomentarius*, *Ganoderma applanatum*. All fungi showed a peak in laccase activity after 30 days of incubation which produced the highest amount of 50 ppm PCP removed. In a static culture study *F. fomentarius* degraded a high amount of PCP during 30 days of incubation, about 96.14%. Fahr et al. (1999) evaluated brown rot fungi *Gloeophyllum striatum* (strains DSM 9592 - DSM

10335) and *Gloeophyllum trabeum* WP 0992 to determinate PCP degradation using radioactively labeled compounds ([U- ^{14}C] PCP). The strains *G. striatum* DSM 9592 and DSM 10335 were tested in a liquid medium contaminated with 5 μM of PCP, but after 19 days of incubation only 10 % of $^{14}\text{CO}_2$ were liberated showing a very slow degradation capacity. Forootanfar et al. (2012) studied the ability of the ascomycete *Paraconiothyrium variable* to eliminate PCP and other CPs in submerged culture medium. The fungal strain was not able to remove 20 mg L^{-1} and PCP minimized the radial and biomass growth.

Among other fungi used in PCP degradation in soil we mentioned Machado et al. (2005) who analyzed the capacity of some basidiomycetes to degrade PCP in soils recovered from areas contaminated with organochlorine industrial residues. Three of the fungi isolated from different ecosystems were tested for tolerance and degradation to high PCP concentrations in soil. The Fungi identified were: *Agrocybe perfecta* CCB161, *Psilocybe castanella* CCB444 and *Peniophora cinerea* CCB204. These species were inoculated into soil containing 1278 mg PCP Kg^{-1} soil supplemented with gypsum and sugar and the authors then evaluated PCP depletion percentages. *P. cinerea* CCB204, *P. castanella* CCB444 and *A. perfecta* CCB161 reduced the PCP present in the contaminated soil by 43, 64 and 78%. All the fungi mineralized PCP, although principally *P. cinerea* CCB204 produced chloride ions during growth in the soil containing PCP, indicating dehalogenation of the molecule. Conversion of PCP to PCA was observed after only 90 days of incubation in the soils inoculated with *A. perfecta* CCB161 and *P. cinerea* CCB204. Rubilar et al. (2007), using a strain of *Bjerkandera adusta* ATTC 90940, carried out a series of PCP remediation laboratory-based studies in slurry soil flasks at initial PCP concentrations of 100, 250 and 350 mg PCP Kg^{-1} soil. *B. adusta* ATTC 90940 degraded PCP no matter the initial PCP concentration and in all cases only 25 mg PCP Kg^{-1} of soil remained. Okeke et al. (1996) determined the temperature, soil moisture potential and initial pH levels might

influence the transformation of PCP by *Lentinula edodes* LE 2. this fungus showed the highest levels of degradation (about 75%) at 25°C and with the soil pH level at 4.0. the extent of PCP degradation related to soil moisture content by *L. edodes* LE 2 was under 50%. using the same strain *L. edodes* LE 2, Okeke et al. (1997) evaluated bioremediation treatment in sterilized and non-sterilized soils contaminated with PCP and inoculated primarily with just the fungus strain and then with *L. edodes* LE2 together with a natural soil micro flora. When *L. edodes* LE2 was used independently, the rate of PCP removal was rapid for the initial 4 weeks and 99% of PCP was biotransformed after 10 weeks. In a mixed culture, PCP biotransformation by *L. edodes* LE2 was slower, only 79% of the PCP was depleted after 10 weeks. PCP and PCA in the soils after 10 weeks were completely eliminated in the sterilized soil with only *L. edodes* LE2, while PCA was still detected in the soils with the mixed micro flora and *L. edodes* LE2. Dechlorination and mineralization of the xenobiotic compound were detected in the presence by *L. edodes* LE2 but the dechlorination efficiency was greater with *L. edodes* LE2 (29.50%) than when fungi were used in a mixed culture (22.40%). Other products were detected from biotransformation of the PCP such as: TeCA and TeCP during the first 4 weeks in both sterilized and non-sterilized soils. Leštan and Lamar (1996) evaluated the PCP degradation in the soil microcosm of organisms such as *Irpex lacteus* ATCC 11245 and *Bjerkandera adusta*. After 4 weeks of incubation in a soil artificially contaminated with 100 µg PCP g⁻¹ soil and inoculated with 3% pellet fungal inoculums, both fungi showed a good capacity to transform PCP to PCA. Besides *I. lacteus* ATCC 11245 and *B. adusta* ATCC 62023 removed 82% and 86% of PCP.

2.5.10. Fungi general discussion

PCP degradation by fungi in a liquid culture has been investigated in a high number of experiments. In these studies, PCP biodegradation focused on the capacity to deplete

PCP using a single fungal strain or a mixed culture. Furthermore, the degradation intermediates products under different conditions and concentrations were analyzed (Table 4). In the liquid cultures, several species of fungi were used, but the genus *Trametes*, *Phanerochaete* and *Anthracophyllum* were surely the most used in this years and capable to degrade PCP (Table 4). These fungi had a capability to remove PCP at any initial concentration ($5 - 300 \text{ mg L}^{-1}$) as it happens so does white-rot fungi (Chiu et al. 1998; Law et al. 2003; Tortella et al. 2008). In the presence of enzymes like laccase, lignin peroxidase and manganese peroxidase there were connections found to higher capacities for PCP degradation. Usually, when fungi increase their enzymatic activity, they showed a higher degradation of PCP (Ryu et al. 2000; Walter et al. 2003; Tortella et al. 2008). When white-rot fungi was inoculated in wood chips or in wheat i.e. lignocellulosic materials, there was a good performance in PCP degradation (Ullah et al. 2000). Lignocellulosic materials are used as vehicles of fungal inoculation in soil or liquid mediums (McAllister et al. 1996) as well as when fungi are used in nitrogen limitation (Aiken and Logan 1996). Less effective were Deuteromycetes and Zygomycetes fungi that only tolerated low PCP concentrations ($< 20 \text{ mg L}^{-1}$), although in a few cases demonstrating a good degradation capacity versus PCP (Cortés et al. 2001; Carvalho et al. 2009). Any type of fungi class that was used in PCP degradation, when the pH level tended to reach acid values, the amount of PCP removed increased considerably (Mathialagan and Viraghavan 2009; Rubilar et al. 2012).

Despite the great ability by some fungi to degrade PCP, there are not many studies on PCP degradation in soil, sediment and sludge (Table 4). In the few works that we have found there are the same species used *in vitro* and all the fungi analyzed fall within the white-rot fungi group. Moreover, little is actually known about the pathway of PCP removal in soil, while, the principal processes that have been conducted on soil contaminated with PCP 1 are known as volatilization, adsorption, leaching and degradation (CCME 1997). The white-rot fungi of the genus *Phanerochaete*,

Anthracophyllum and *Trametes* are surely the most frequently used as well as being the most efficient microorganism in PCP bioremediation experiments in soil; as stated in the previous paragraph. The principal compounds product by PCP biodegradation for the three fungi was PCA (Leštan and Lamar 1996; Tuomela et al. 1999; Machado et al. 2005; Rubilar et al. 2007). They were very adept at degrading large amounts of PCP (250 – 1000 mg Kg⁻¹ soil) as well as *Lentinula edodes* LE2 (~200 mg Kg⁻¹), *Agrocybe perfecta* CCB161, *Psilocybe castanella* CCB444 and *Peniophora cinerea* CCB204 (~1278 mg Kg⁻¹). The remaining fungi analyzed were able to degrade PCP only at an initial concentration of < 100 mg Kg⁻¹ soil. All the fungi that were analyzed in these studies that exploited lignin peroxidase, manganese peroxidase and laccase were able to degrade PCP and convert it essentially into PCA or TCHQ through dehalogentaion and dechlorination (McAllister et al. 1996; Cea et al. 2005; Field and Sierra-Alvarez 2008; Rubilar et al. 2011) (Table 4). The ability for PCP degradation was increased when the fungi were incorporated in lignocellulosic materials such as wheat grains (Rubilar et al. 2011), transformed into pellets (Leštan and Lamar 1996), adjusting the pH levels (4-5.5) and temperatures (25 °C) (Okeke et al. 1996; Rubilar et al. 2011) or by inserting a contaminated soil gypsum and sugar (Machado et al. 2005). An important role in the removal of PCP is that of the indigenous microorganisms. These last, in some experiments have showed a natural ability to degrade or adsorb PCP in soil at high percentages (<50%) (Okeke et al. 1996; Cea et al. 2005; Rubilar et al. 2011). For example, in the Chilean andisols 40 % of was removed due to their specific characteristics and ability to sorbent PCP and other CPs, all thanks to their allophane–ferrihydrite association with organic matter (Cea et al. 2005). Organic matter shows a very good efficiency at adsorbing PCP (Scelza et al. 2008) that in a moment can then be degraded by autochthonous microorganisms (Okeke et al. 1996).

Table 4. Degradation of PCP by fungi

Species	Conditions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
<i>Phanerochaete chrysosporium</i> ATCC 42725	Soil	100 µg g ⁻¹	+	NR	NR	PCA; 2,3,4,6-tetrachloroanisole	Leštan and Lamar 1996
<i>Phanerochaete sordida</i> HHB-8922-Sp	Soil	100 µg g ⁻¹	92%	NR	NR	PCA; 2,3,4,6-tetrachloroanisole	
<i>Irpex lacteus</i> ATCC 11245	Soil	100 µg g ⁻¹	82%	NR	NR	PCA; 2,3,4,6-tetrachloroanisole	
<i>Bjerkandera adusta</i> ATCC 62023	Soil	100 µg g ⁻¹	86%	NR	NR	PCA; 2,3,4,6-tetrachloroanisole	Okeke et al. 1996
<i>Trametes versicolor</i> MD-277	Soil	100 µg g ⁻¹	86%	NR	NR	PCA; 2,3,4,6-tetrachloroanisole	
<i>Lentinula edodes</i> LE2	Soil	200 mg Kg ⁻¹	50 - 70%	NR	NR	NR	
<i>Phanerochaete chrysosporium</i> BKM 1767	Soil	200 mg Kg ⁻¹	75 - 85%	NR	NR	NR	Aiken and Logan 1996
<i>Phanerochaete chrysosporium</i> BMK-F-1767	Liquid	250 mg L ⁻¹	72 - 95%	NR	Chloride ions	Chloride ions	
<i>Lentinula edodes</i> LE2	Soil	200 mg Kg ⁻¹	35%	+	10%	PCA	
<i>Phanerochaete sordida</i>	Soil	175 ppm	+	NR	+	PCA	Pfender et al. 1997
<i>Trametes versicolor</i>	Liquid	25 mg L ⁻¹	>99%	NR	NR	NR	Pallerla and Chambers 1998
<i>Armillaria gallica</i> 1039	Liquid	25 mg L ⁻¹	+	NR	NR	NR	
<i>Armillaria gallica</i> 1057	Liquid	25 mg L ⁻¹	+	NR	NR	NR	
<i>Armillaria mellea</i> M51	Liquid	25 mg L ⁻¹	+	NR	NR	2-methyl-1,3 benzenediol; 6-phenyl-dodecane	Chiu et al. 1998
<i>Ganoderma lucidum</i> HK-1	Liquid	25 mg L ⁻¹	+	NR	NR	2-methyl-1,3 benzenediol	
<i>Lentinula edodes</i> L54	Liquid	25 mg L ⁻¹	+	NR	NR	2-methyl-1,3 benzenediol; 1-octyl-benzene	
<i>Lentinula edodes</i> L67	Liquid	25 mg L ⁻¹	+	NR	NR	NR	
<i>Lentinula edodes</i> L68	Liquid	25 mg L ⁻¹	+	NR	NR	NR	
<i>Phanerochaete chrysosporium</i> M1	Liquid	25 mg L ⁻¹	+	NR	NR	1-chloro-3-methoxy-benzene	
<i>Pleurotus pulmonarius</i> PL-27	Liquid	25 mg L ⁻¹	+	NR	NR	3,3-dimethyl-cyclohexanol	Tuomela et al. 1999
<i>Polyporus</i> sp. Cv-1	Liquid	25 mg L ⁻¹	+	NR	NR	NR	
<i>Volvariella volvacea</i> V34	Liquid	25 mg L ⁻¹	+	NR	NR	1-chloro-3-methoxy-benzene	
<i>Trametes versicolor</i> PRL 572	Soil	996 µg g ⁻¹	29%	NR	NR	benzenediol	Ullah and Evans 1999
<i>Coriolus versicolor</i>	Liquid	50 - 200 ppm	+	NR	+	PCA; 2,3,4,6-tetrachloroanisole	
<i>Gloeophyllum striatum</i> DSM 9592	Liquid	5 µM	10%	NR	NR	NR	
<i>Gloeophyllum striatum</i> DSM 9592	Liquid	5 µM	10%	NR	NR	NR	Ryu et al. 2000
<i>Phanerochaete chrysosporium</i> IFO 31249	Liquid	30 mg L ⁻¹	72.6%	NR	NR	NR	
<i>Trametes</i> sp. KFCC 10941	Liquid	30 mg L ⁻¹	64%	NR	NR	NR	
<i>Pleurotus</i> sp. KFCC 10943	Liquid	30 mg L ⁻¹	70.33%	NR	NR	NR	Ullah et al. 2000
<i>Coriolus versicolor</i> FPRL-28A	Liquid	50 - 100 ppm	75 - 100%	NR	NR	NR	
<i>Phanerochaete chrysosporium</i> OGC101	Liquid	100 M	10 - 90%	+	+	NR	
<i>Rhizopus nigricans</i>	Liquid	12.5 - 500 mg L ⁻¹	+	NR	NR	NR	Reddy and Gold 2000
<i>Rhizopus nigricans</i>	Liquid	12.5 mg L ⁻¹	60 - 100%	NR	NR	NR	
<i>Trichoderma harzianum</i> 2023	Liquid	10 ppm	+	NR	NR	NR	
<i>Asbidia fusca</i>	Liquid	100 mg L ⁻¹	35 - 40 %	NR	NR	NR	Guiraud et al. 2003
<i>Mucor ramosissimus</i> IM 6203	Liquid	10 mg L ⁻¹	+	NR	NR	2,3,5,6-TCHQ	
<i>Abortiporus biennis</i> HR 145	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Oudemansiella australis</i> HR345	Liquid	50 mg L ⁻¹	+	NR	NR	NR	Walter et al. 2003

Table 4. Continued

Species	Conditions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
<i>Peniophora sacra</i> HR226	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Peniophora sacra</i> HR235	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Peniophora sacra</i> HR240	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Peniophora sacra</i> HR241	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Rigidoporus catenarius</i> HR316	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Stereum fasciatum</i> HR348	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes</i> sp. HR192	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes</i> sp. HR196	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes</i> sp. HR197	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes versicolor</i> HR131	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes versicolor</i> HR154	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes versicolor</i> HR160	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes versicolor</i> HR275	Liquid	50 mg L ⁻¹	100%	NR	NR	NR	
<i>Trametes versicolor</i> HR277	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Trametes versicolor</i> HR445	Liquid	50 mg L ⁻¹	+	NR	NR	NR	
<i>Pleurotus pulmonarius</i>	Liquid	2 - 100 ppm	60 - 90%	NR	NR	TCHQ ; TCP	Law et al. 2003
<i>Agrocybe perfecta</i> CCB161	Soil	1180 - 1278 mg Kg ⁻¹	78%	NR	+	PCA ; Chloride ions	Machado et al. 2005
<i>Trametes villosa</i> CCB176	Soil	1180 - 1278 mg Kg ⁻¹	58%	NR	+	Chloride ions	
<i>Trametes villosa</i> CCB213	Soil	1180 - 1278 mg Kg ⁻¹	58%	NR	+	PCA ; Chloride ions	
<i>Psilocybe castanella</i> CCB444	Soil	1180 - 1278 mg Kg ⁻¹	64%	NR	+	Chloride ions	
<i>Peniophora cinerea</i> CCB204	Soil	1180 - 1278 mg Kg ⁻¹	43%	NR	+	PCA ; Chloride ions	
<i>Penicillium camemberti</i>	Liquid	0.001M	+	NR	NR	Chloride ions	Taseli and Gokcay 2005
<i>Phanerochaete chrysosporium</i> BKM-F-1767	Soil	100 mg Kg ⁻¹	90%	NR	NR	NR	Jiang et al. 2006
<i>Trametes versicolor</i> HR131	Soil	1000 mg Kg ⁻¹	> 90%	NR	NR	PCA	Walter et al. 2005
<i>Anthracoophyllum discolor</i>	Soil	100 - 300 mg Kg ⁻¹	50 - 100%	NR	NR	PCA ; TCHQ	Rubilar et al. 2007
<i>Bjerkandera adusta</i> ATTC 90940	Soil	100 - 300 mg Kg ⁻¹	> 80%	NR	NR	PCA ; TCHQ	
<i>Byssocladium fulva</i>	Liquid	12.5 - 25 mg L ⁻¹	20%	NR	NR	NR	Scelza et al. 2008
<i>Anthracoophyllum discolor</i> Sp4	Liquid	25 mg L ⁻¹	96%	NR	NR	NR	Tortella et al. 2008
<i>Lenzites betulina</i> Ru-30	Liquid	25 mg L ⁻¹	80%	NR	NR	NR	
<i>Inonotus</i> sp. Sp2	Liquid	25 mg L ⁻¹	< 50%	NR	NR	NR	
<i>Stereum</i> sp. Ru-24	Liquid	25 mg L ⁻¹	< 50%	NR	NR	NR	
<i>Phanerochaete chrysosporium</i> CECT-2798	Liquid	25 mg L ⁻¹	72%	NR	NR	NR	
<i>Galerina patagónica</i> Sp3	Liquid	25 mg L ⁻¹	88%	NR	NR	NR	
<i>Stereum hirsutum</i> Sp1	Liquid	25 mg L ⁻¹	< 50%	NR	NR	NR	
<i>Stereum hirsutum</i> Ru-104	Liquid	25 mg L ⁻¹	< 50%	NR	NR	NR	
<i>Trametes hirsuta</i> Ru-008	Liquid	25 mg L ⁻¹	0%	NR	NR	NR	
<i>Trametes versicolor</i> Ru-107	Liquid	25 mg L ⁻¹	0%	NR	NR	NR	

Table 4. Continued

Species	Conditions	PCP	Degradation	Dechlorination	Mineralization	Product(s)	References
<i>Trametes versicolor</i> Ru-0030	Liquid	25 mg L ⁻¹	0%	NR	NR	NR	Montiel-González et al. 2009 Ramesh and Pattar 2009
<i>Anzylomyces rouxi</i>	Liquid	12.5 mg L ⁻¹	50 - 100%	NR	NR	NR	
<i>Laetiporus cincinnatus</i>	Liquid	50 ppm	+	NR	NR	NR	
<i>Trametes versicolor</i>	Liquid	50 ppm	96.14%	NR	NR	NR	
<i>Fomes fomentarius</i>	Liquid	50 ppm	+	NR	NR	NR	Carvalho et al. 2009
<i>Ganoderma aplanatum</i>	Liquid	50 ppm	+	NR	NR	NR	
<i>Pleurotus ostreatus</i>	Liquid	50 ppm	+	NR	NR	NR	
<i>Chrysomilla siophila</i> DSM 16514	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	
<i>Mucor plumbeus</i> DSM 16513	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	Szewczyk and Dlugonski 2009
<i>Trichoderma longibrachiatum</i> DSM 16517	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	DCBQ	
<i>Cladosporium herbarum</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	DCBQ	
<i>Penicillium glabrum</i> DSM 16516	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	
<i>Penicillium osonii</i> DSM 16515	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	Cea et al. 2010
<i>Eupenicillium hirayanae</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	
<i>Penicillium brevicompactum</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	
<i>Penicillium glandicola</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	
<i>Penicillium variabile</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	González et al. 2010
<i>Penicillium diversum</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	
<i>Penicillium decumbens</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	DCBQ	
<i>Penicillium janczewskii</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	
<i>Penicillium corylophilum</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	CHQ	de Souza et al. 2011
<i>Penicillium adametzi</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	TeCBQ	
<i>Penicillium fennelliae</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	
<i>Penicillium restrictum</i>	Liquid	1 - 20 mg L ⁻¹	+	NR	NR	NR	
<i>Mucor ranonissimus</i> IM 6203	Liquid	10 mg L ⁻¹	90%	NR	NR	2,3,5,6-TCHQ ; pentachloromethoxybenzene	Szewczyk and Dlugonski 2009
<i>Anthracytophyllum discolor</i>	Soil	250 mg Kg ⁻¹	93.6%	NR	NR	NR	Cea et al. 2010
<i>Trametes pubescens</i> CBS 696.94	Liquid	30 mg L ⁻¹	77%	NR	NR	Chloride ions	González et al. 2010
<i>Pleurotus pulmonarius</i> CCB19	Liquid	25 mg L ⁻¹	20 - 70%	NR	NR	NR	de Souza et al. 2011
<i>Anthracytophyllum discolor</i>	Soil	250 - 350 mg PCP Kg ⁻¹	80 - 93%	NR	NR	NR	Rubilar et al. 2011
<i>Phanerochaete chrysosporium</i> CECT-2798	Soil	250 - 350 mg PCP Kg ⁻¹	65 - 79%	NR	NR	NR	León-Santestebán et al. 2011
<i>Rhizopus oryzae</i> ENHE	Liquid	12.5 - 25 mg L ⁻¹	85 - 88%	NR	NR	NR	
<i>Mucor plumbeus</i> DSM 16513	Liquid	15 - 18.8 µM	+	+	+	TriCHQ ; TCHQ	
<i>Trametes pubescens</i> CBS 696.94	Liquid	15 mg L ⁻¹	41%	NR	NR	NR	
<i>Anthracytophyllum discolor</i> Sp4	Liquid	20 - 50 mg L ⁻¹	NR	NR	NR	NR	Gaitan et al. 2011
<i>Paraonothyrium variabile</i>	Liquid	20 mg L ⁻¹	0%	NR	NR	NR	Rubilar et al. 2012
							Forootanfar et al. 2012

NR: Not reported ; +; denotes positive observation ; - denotes no response

2.6. Concluding remarks

How quickly, completely and efficiently PCP is degraded, depends on microorganism biodiversity and environmental conditions. Several bacteria and fungi have the capability to biodegrade PCP and they have been isolated from a variety of environments: industrial sewage (Szewczyk et al. 2003; Machado et al. 2005), contaminated soils, and effluent and fresh water sediments (Shah and Thakur 2003; Chandra et al. 2006; 2009; Lin et al. 2008). PCP degradation, mineralization, adsorption and dechlorination by microorganisms in different conditions are summarized in tables 3 and 4. Analyzing all the works of PCP bioremediation, we have found that aerobic microorganisms have been shown to be highly efficient at degrading and mineralizing at higher PCP concentrations, more than the anaerobic microorganisms. Furthermore, bacteria showed the greatest efficiency, in regards to, degradation of PCP, whereas the fungi demonstrated lower capability and efficiency. In addition, all the bacteria and fungi, in a pure or mixed culture, act much better when used with an amendment (wheat, wood chips, glucose). However, bacteria and fungi in mixed cultures have been shown to completely degrade PCP, with a pre exposition to PCP, the biodegradation capacity significantly increased. Pellets, immobilizing cells and engineered systems also were much more efficient in the degradation of PCP. Finally, exploiting local biodiversity with the biostimulation of the microbial community with compost or soil improvers, the capacity to degrade the PCP increase significantly in time (Schmidt et al. 1990; Alber et al. 2000; Puglisi et al. 2009)

In this review, numerous genus of bacteria were studied that can utilize PCP as carbon and energy sources such as: *Pseudomonas* sp., *Flavobacterium* sp., *Mycobacterium* sp., *Sphingomonas* sp., *Kokuria* sp., *Bacillus* sp., *Serratia* sp. and *Arthrobacter* sp. PCP biodegradation by these bacteria is well established and most of the studies have evaluated the metabolism and co-metabolism with unsubstituted or substituted PCP as the primary substrate. The main strategies and processes used with bacteria in the

degradation of PCP released intermediate products such as TCHQ, TriCHQ, CHQ, DCHQ, TCC, CHYQ and 2,3,4,6-TeCP (McAllister et al 1996; Shah and Thakur, 2002; Sharma and Thakur 2008; Field and Sierra-Alvarez 2008; Singh et al. 2008; Singh et al. 2009; Chandra et al. 2009). Several Gram-negative and positive bacterial strains were used in PCP remediation, even if, Gram-negative strains seem to be more efficient in the tolerance and degradation of PCP (McAllister et al 1996; Field and Sierra-Alvarez 2008). This happens to be because these bacteria are able to exclude PCP from the cell and due to the presence of lipopolysaccharide in the cell wall (Izaki et al. 1981). Aerobic and anaerobic biodegradation of PCP by the bacterial strains has been demonstrated in field and laboratory works. As well as the genes that produce useful enzymes for PCP degradation in *Flavobacterium* sp. ATCC39723 which have been characterized and cloned in *Escherichia coli*, granting the latter with the ability to degrade PCP (McAllister et al. 1996). In fact, the characteristics of PCP degradative enzymes can be improved by engineering methods to move forward their potential in the bioremediation strategy and in industrial applications. Cloning in *Pseudomonas gladioli* genes of *Sphingomonas chlorophenolicum* (Dai and Copley 2004) and some genes of *Phanerochete chrysosporum* in *Amylomyces rouxi* (Stewart et al 1996), these authors observed significant improvements in the rate and capacity of these organisms to degrade PCP. Therefore cloning the genes that useful in the degradation of PCP into the indigenous microorganisms that, for example, don't have the capacity to degrade contaminants could overcome some problems related to introduction into soils or other mediums producing new "exotic" organisms. However little is yet known on the potential pathogenic effect that any species can produce versus other microorganisms, like plants and animals. There are some species such as *Pseudomonas aeruginosa* and *Bacillus cereus* that for example are very dangerous for human health (He et al. 2004; Kotiranta et al. 2000). This fact may greatly limit their use in the bioremediation strategy.

Fungi as well are very useful as PCP degraders, especially the genus: *Phanerochaete*, *Anthracyllum*, *Agrocybe*, *Lentinula* and *Trametes*. Different genus or families of fungi exhibit tolerances and degradation capacities to PCP. Furthermore, fungal strains are generally less efficient than bacteria in PCP degradation and only in only a few cases are they able to completely mineralize PCP. In addition, fungi adsorb PCP on the mycelium, leaving intact the contaminant. However, the fungi strains, thanks to their excellent enzymatic pool, can break down PCP in a molecule, making them more bioavailable to be degraded by other microorganisms (McAllister et al. 1996; Pointing 2001). Unlike bacteria, fungi are not capable of using PCP as a source of carbon. PCP degradation is not a direct consequence of fungal metabolism, but rather of a co-metabolic process. They have enzyme systems useful in degrading wood components such as lignin o cellulose i.e. PH-oxidase, laccase lignin and manganese peroxidase, which are capable of breaking down PCP molecules (McAllister et al. 1996; Pointing 2001). For fungi, the main strategies and processes in the degradation of PCP release intermediate products such as PCA, TCHQ, TCP, CHQ, DCBQ and TriCHQ (Leštan and Lamar 1996; Machado et al. 2005; Carvalho et al. 2009; Rubilar et al. 2009; Carvalho et al. 2011). While for white or brown-rot fungi, a future in bioremediation is defiantly possible, because they are not dangerous to humans or animals; although some species such as *Armillaria mellea* can cause serious diseases in many plants i.e. if there is a bioremediation intervention in an agricultural field. Therefore, it also depends on the context in which the fungi isolates will be used. For some deuteromycetes and zigomycetes species, like some bacteria, little is yet known on their potential pathogenic effects on other microorganisms or versus plants and animals; for example the species *Rhizopus oryzae* that can cause oral or cerebral mucormycosis. PCP toxicity is well known fact, especially for some organisms (Crosby 1981); the toxicity of the degradation intermediate products is still not well documented. In fact, microbial

metabolism of contaminants such as PCP may produce, in some cases, toxic metabolites (McAllister et al. 1996; Field and Sierra-Alvarez 2008).

Improving the biodiversity of the microorganisms present in a medium, a pollutant compound can be completely and more quickly mineralize. In fact it is important to remember that increasing microbial biodiversity, we increase the possibility to have organisms capable to degrade the contaminant or its intermediates products. Microorganisms use essentially oxygenases and hydroxylase that insert O_2 and $-OH$ into the compound prior to ring cleavage or using reductive dechlorination, which eliminate a Cl-group at the compounds ring, which is essential to the intermediate metabolites in an aerobic pathway of PCP degradation (McAllister et al. 1996; Field and Sierra-Alvarez 2008). But in bioremediation strategy it is equally important to know the environmental conditions (physical and chemical properties of the sites) and physico-chemical characteristics of the contaminant (Providenti et al. 1993). It is essential to know as well the relation and interaction between microbial consortium - environmental conditions - toxic compounds, since this will allow the researcher to obtain high performances in the bioremediation process i.e. to achieve degradation, bioaccessibility and bioavailability of PCP, some factors such as aeration, moisture, content of the organic matter, microbial biodiversity, temperature and pH level, soil improvers and compost could be optimized PCP degradation (Providenti et al., 1993; McAllister et al. 1996; Scelza et al. 2008; Puglisi et al. 2009; Cea et al. 2010; Juwarkar et al. 2010).

2.7. Future perspectives

All the information occurs in this review can be used to push forward the recent bioremediation technological advances such as “omic” based technique (genomics, proteomics and metabolomics).

Understudying thoroughly as the microorganisms can tolerate, degrade and mineralize some pollutants as PCP, represent the first step to realize an excellent remediation processes/studies. Reviewing the case studies showed in this work could be further increased the experiments to extend the knowledge of genes encoding of the metabolites useful in PCP degradation. Until now the molecular aspect of PCP degradation have received little attention (Orser and Lange 1994; Juwarkar et al. 2010; Villemur 2013; Carvalho et al. 2013; Copley et al. 2013). Moreover the genetic manipulation can offer a means of engineering microorganism to deal with PCP that may be present in a contaminated site (Villemur 2013). Other experiments should be carried out about how PCP effect microbial cell. It is little know how PCP can influences not only microorganisms community but also more specifically cellular process, production of toxins, cyclic changes in morphology, lipid membrane components, biomass growth, enzymatic activity, sporulation and reproduction capacity. Another interesting focal point worthwhile to examine is the microbial interaction in relation to the PCP. It could increase the possibility to use allochthonous microorganisms in bioremediation processes. This point is currently highly debated because many authors believe that to insert a microorganism, for example in a different soil, generates a turnover in microbial community. This is absolutely true, but the soil, like the water and air, is a dynamic system constantly changing and allochthonous microorganisms are continuously transported by wind, animal and rain in different areas even thousands of miles away.

It is very important to know the interaction of all the factors dealing the bioremediation process as well as reforming and restructuring the strategy in which contaminated sites are processed and effectively decontaminated. In this way we can move the world forward to produce a safer environment for human, animal and plant communities.

2.8. References

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Anthracophyllum discolor

3. Effect of pentachlorophenol concentration and pH levels on the adsorption capacity of *Anthracophyllum discolor* pellets

3.1. Abstract

Pentachlorophenol (PCP) is an extremely dangerous pollutant for every ecosystem as well as the health of many organisms. It has been introduced into the environment, primarily, as a wood preservative. Even so, the indiscriminate use of PCP has led to the contamination of water and soil systems. In this study we have detected that the PCP concentration and pH levels can influence the PCP adsorption of live pellets of *Anthracophyllum discolor* for such purposes as the bioremediation of PCP contaminated water. PCP adsorption was evaluated after 24 hours in KCl 0.1 M electrolyte solution with initial PCP concentrations of 5 and 10 mg L⁻¹ and with pH values between 4 and 9 (at intervals of 0.5). The Fourier Transform Infrared Spectroscopy (FTIR) was used to identify functional groups of fungal biomass that can interact with PCP. Fungal growth and enzyme production were examined after the adsorption experiment. The PCP that was adsorbed by *A. discolor* was >80% compared to pH values of 5 and 5.5, which were the two concentrations being analyzed. PCP adsorption significantly decreased in liquid medium of pH > 6.0. FTIR results showed that amides, alkanes, carboxylates, carboxyl and hydroxyl groups may possibly be important to the PCP adsorption for pellets of *A. discolor*. Contrarily, amino and phosphate groups potentially do not have important roles in the PCP adsorption process. The two PCP concentrations used in our experiments, after 24 hours, did not negatively affect the mycelium growth, nor did it degrade the fungi enzyme production. The live pellets of *A. discolor* may be used as a natural biosorbent for liquid solutions which are contaminated by PCP.

3.2. Introduction

Pentachlorophenol (PCP) is considered to be one of the most hazardous contaminants of soil and water. It was widely used for many years in different areas of the world as a low cost biocide (EPA 1984). Some of the more large scale uses of this contaminate was in the making of most wood products. The wood industry used PCP for the preservation of timber and the bleaching of paper or tissues (EPA 1984; ATSDR 2001). PCP is classified as a priority pollutant in the USA and Europe for its high toxicity. To deplete this toxic compound from environment, the most common processes used today are: adsorption with activated carbon, scorched in an approved secure area and closed in sealed containers, washed with chemical products, incineration at high temperatures for a long period of time (ATSDR 2001; EPA 2008). A further low-cost method to remove PCP is the biological treatment described as microbial degradation (McAllister et al. 1996; Gadd 2001; Singh 2006; Rubilar et al. 2008; Field and Sierra-Alvarez, 2008) and adsorption (Aksu 2005; Ahmaruzzaman 2008).

Removal of PCP by adsorption of live or dead microorganism uses the biomass capacity which may be used as an effective adsorbent for the particular attraction between the cellular component and the pollutant compound. The use of microorganisms for PCP adsorption is increasing because offers an economical, practical and efficient alternative to the commonly used methods (Ahmaruzzaman 2008). Recent literature has shown many microorganisms that have proven to be performing in PCP adsorption. Mathialagan and Viraraghavan (2009) showed that the non-viable biomass of *Aspergillus niger* was pH-dependent in PCP adsorption. Moreover, *A. niger* biomasses treated with cetyltrimethylammonium showed a 100% removal at 1 mg PCP L⁻¹ to all pH levels from the aqueous solutions. Brandt et al. (1997) detected that *Mycobacterium chlorophenolicum* can effectively be used in PCP

adsorption. Jianlong et al. (2000) obtained better results in regards to the biosorption ability of PCP by the microbial biomass in an aqueous solution. Fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ganoderma lobatum*, *Inonotus dryophilu* and several other species were used by Logan et al. (1994) in an experiment of PCP adsorption using dead biomass. All species showed a good adsorption capacity at acidic pH levels. Rubilar et al. (2012) also used dead biomass from the white-rot fungus reactor *Anthracophyllum discolor* in a fixed-bed column reactor for PCP adsorption at different concentrations and pH values. It was concluded that *A. discolor* dead biomass was an excellent adsorbent for PCP at acid pH values.

Almost all studies in pollutant adsorption using biomass are often developed with dead biomass. This happens because dead microbial cells do not provide toxicity concerns and do not require growth media or nutrients. Moreover, this biomass can be stored for a long time and be used in several cycles of adsorption and desorption of the pollutant compound. However, live biomass have, in many cases, shown better performances in pollutant adsorption than dead biomass. In live microorganisms the adsorption and biodegradation processes can be used together to help the contaminant removal (Logan et al. 1994 ; Benoit et al 1998 ; Damianovic et al. 2009). Furthermore, there are few studies that are focused on the effect of the pH levels and the PCP concentrations on adsorption capacity of live fungal biomass.

The principal objective of the present study is to evaluate the adsorption capacity of pellets by *A. discolor* in a liquid medium which can be affected by different pH values and initial PCP concentrations. FTIR spectroscopy analysis was carried out on *A. discolor* biomass to find functional groups that are active during PCP adsorption. Finally, we evaluated if the initial PCP concentration negatively influenced the mycelium growth and the fungi enzyme production.

3.3. Materials and Methods

3.3.1. Fungal strain and cultivation conditions

Anthracoophyllum discolor is a white-rot fungus isolated from carpophores collected from decayed wood in the temperate forest of southern Chile (Tortella et al. 2008). The strain was stored on malt extract agar (MEA) (15 g L^{-1} agar; 3.5 g L^{-1} malt extract; 10 g L^{-1} glucose) in slant tubes at 4°C in the Environmental Biotechnology Laboratories of the Universidad de La Frontera (Temuco, Chile).

3.3.2. Preparation of the fungal pellets

Seven plugs (diameter 6 mm) of *A. discolor* mycelium cultured on MEA for 7 days in Petri dishes, were placed in a flask which contained 100 ml of modified Kirk medium (10 g L^{-1} glucose; 2 g L^{-1} peptone; 2 g L^{-1} KH_2PO_4 ; 0.5 g L^{-1} MgSO_4 ; 0.1 g L^{-1} CaCl_2 ; 3.3 g L^{-1} sodium acetate; 5 ml L^{-1} tween 80 to 10 %; 2.11 ml L^{-1} MnSO_4) and incubated at 25°C for 7 days. The fungal mycelium in the culture broth was homogenized with a blender for 2 minutes to prepare the inoculum. Finally, 2 ml of inocula (1.5 mg L^{-1} fungus dry weight) were placed in the flasks which contained 100 mL of modified Kirk medium and incubated at 25°C on a rotating shaker incubator at 120 r.p.m. for 10 days in order to obtain the pellets. The formed pellets were washed with distilled water and stored in an empty falcon at 25°C for 3 days.

3.3.3. Surface area measurement

The specific surface area of the lyophilized pellets of *A. discolor* was determined by the B.E.T. surface area analyzer (Nova 1000e – Surface area & Pore size analyzer). The

dried biomass was initially degassed by evacuation for 2 h at 80 °C, and then the surface area was determined by the N₂ adsorption method (He and Tebo 1998).

3.3.4. Potentiometric titration of *A. discolor* cell wall

The Surface charge behavior was assessed by acid-base titration in a N₂ atmosphere using 0.01 N NaOH and 0.01 N HCl. An amount of 20 *A. discolor* pellets (20 ± 0.2 mg fungus dry weight) were added to two teflon vessels containing 100 ml of 0.001, 0.01 or 0.1 M KCl as a background solutions. The titrations were carried out by adding 200 µl of titrant after 15 minutes reaction time between additions to allow stabilization of the pH. To measured the pH levels was used the pH meter Thermo Scientific Orion 3-Star. This instrument accurately measure pH to 2 decimal points. Finally, the surface charge behavior of the fungus was evaluated in the presence of PCP at two concentrations (5 and 10 mg L⁻¹) using KCl 0.1 M as a background electrolyte.

3.3.5. PCP adsorption

PCP adsorption experiments were carried out in 10 ml amber glass bottles to determine the adsorption capacity of pellets as a function of pH levels. In each bottle 5 mL of 0.1 M KCl (as background electrolyte) was added with either of the two PCP concentrations (5 and 10 mg L⁻¹) as well as 20 pellets of *A. discolor*. The pH levels of the solutions were adjusted with HCl or NaOH (0.1 M) and stabilized from a level of 4 to a level of 9 at intervals of 0.5 unit of pH. The control was carried out in the same procedure described previously only without the addition of PCP. The bottles were incubated at 25°C on a rotating shaker incubator at 120 r.p.m. for 24 h in darkness to avoid PCP photodegradation. The assays were conducted in triplicate. After incubation the samples were analysed for pH levels. The amount of PCP adsorbed was determined by the difference of PCP added and the residual PCP present in the solution after 24 hours. An aliquot (1 mL) of the liquid phase was filtered with the Syringe Filters

Chromotech (pore size 0.45 μm ; filter size 25 mm) and analyzed by using High Performance Liquid Chromatography (Hitachi Lachrom Elite) by way of a Chromolith RP-18e, 100 mm x 4.6 mm column. The injection volume was 20 μL . The mobile phase consisted of acetonitrile and phosphoric acid (1% aqueous solution) 1:1 (v/v) with a flow rate of 1 mL min^{-1} . Instrument calibration and quantifications were performed against the pure reference standard (5 and 10 mg L^{-1}). The detector wavelength was set at 215 nm with a retention time of 4.5 minutes.

3.3.6. Fourier transforms Infrared spectroscopy

The analysis of the fungal pellets were performed by FTIR only after the adsorption of PCP. Briefly, 20 *A. discolor* pellets were added to a flask with 100 mL of 0.1 M KCl and 10 mg L^{-1} PCP. A control solution without PCP was obtained. The flasks were incubated at 25°C on a rotating shaker incubator at 120 r.p.m. for 24 h. The fungal biomass was separated from the KCl medium by using filter paper (Whatmann no.1), washed with distilled water and dried in an oven at 30°C for 24 h. The samples of 1 mg were mixed with 100 mg of KBr. The FT-IR spectrum was achieved by using a Bruker Tensor 27 with the following parameters: resolution 2 cm^{-1} ; 32 scans min^{-1} ; transmittance spectrum from 4000 cm^{-1} to 500 cm^{-1} ; open setting 6 mm; scanner rate 10 KHz.

3.3.7. Assessing *A. discolor* growth and Remazol Brilliant Blue R decolorization

A. discolor pellets were recovered from the amber bottles to evaluate the alterations in the fungal growth and production of enzymes after the 24 hours of PCP adsorption (5 or 10 mg L^{-1}) or no-adsorption (control). The differences in the mycelium growth (cm day^{-1}) of *A. discolor* pellets was measured in a culture of potato dextrose agar (PDA) (15 g L^{-1} agar; 20 g L^{-1} dextrose; 4 g L^{-1} potato extract) in Petri dishes. The fungal

pellets were incubated at 25 °C for 5 days in darkness. The peroxidase activity was performed in Petri dishes (80 mm diameter) according to Eichlerová et al. (2006). The medium contained agar (15 g L⁻¹), malt extract (3.5 g L⁻¹), glucose (15 g L⁻¹) and Remazol Brilliant Blue R (0.2 g L⁻¹) and was aseptically transferred in Petri dishes. These latter were inoculated with one *A. discolor* pellet and incubated at 25 °C for 7 days in darkness. The Remazol Brilliant Blue R dye turns from blue to yellow opaque as it is decolorized by peroxidase of fungus.

3.3.8. Statistic analysis

Every experiment was conducted in triplicate and the mean value is represented by one data point in the figures. All data were subjected to ANOVA by using XLSTAT 2013.1 for Windows. The assumptions of normality and homogeneity of the variants were tested by the Kolmogoroff–Smirnov. The significant differences between means of $P < 0.05$ were assessed in accordance with Tukey's multiple comparison test.

3.4. Results and Discussion

3.4.1. Morphological characteristics of *A. discolor* pellets

PCP adsorption was evaluated using pellets of *A. discolor* with the following morphological characteristics: diameter 2.57 ± 0.47 mm, weight 1.53 ± 0.51 mg and a surface area of 1.421 ± 0.6 m² g⁻¹. For the biotechnological industry, the fungal pellets may offer efficient and practical alternatives to other techniques currently used to remove this pollutant from soil and water. Moreover, the interest in the potential use of these pellets as biosorbents is favorable for their simplicity of reuse as well as being economically advantageous (Lin et al. 2008; Kumar et al. 2009). The fungal adsorption studies were almost all carried out with dead biomass (Kumar et al. 2008; Mathialagan and Viraraghavan 2009 ; Rubilar et al. 2012). It has been widely proven that with dead

biomass there are no toxicity concerns and maybe eventually reused for numerous cycles of adsorption. Tsezos and Bell (1989) demonstrated as PCP adsorption by dead biomass of *Rhizopus arrhizus* was six times higher than the respective live biomass. However, using live microorganisms, over biosorption, also the biodegradation processes can help resulted in a better and faster depletion of the contaminant (Logan et al. 1994; Benoit et al. 1998; Damianovic et al. 2009).

3.4.2. Potentiometric titration

The Potentiometric titration curve (Fig. 1) maybe observed as the pH level of the solution increases, the net surface charge becomes more negative as demonstrated by Deng et al. (2009) and Mathialagan and Viraraghavan (2009). At a pH of > 7.0 , the net charge of the fungal wall was negative. The point where the line representing different ionic strengths intersect is referred to the point of zero salt effect (PZSE). The potentiometric titration curve of *A. discolor* pellets, for all KCl solutions, showed pronounced flexes as in Navarro et al. (2008). This allows us to locate the area of equivalence points indicating the neutralization of different acidic groups, since the acidity of the latter varies with pH. This area was very evident, contrary to what was observed with *Penicillium chrysogenum* (Skowronski et al. 2001; Deng et al. 2009), *Trichoderma viride* (Sanna et al. 2002), *Spirulina platensis* (Lodi et al. 2003), *Rhizopus arrhizus* (Naja et al. 2006) and in humic and fulvic acids (Stevenson, 1977). The potentiometric titration curves obtained in the presence of two PCP concentrations are shown in Fig. 2. The PZC shifted to a lower pH value with increasing PCP concentrations (PZC control at pH 7.0; PZC 2.5 mg PCP L⁻¹ at pH 5.82; PZC 5 mg PCP L⁻¹ at pH 5.10). This could indicate the possibility of an inner-sphere complex formation due to a release of H⁺ from the protonated anions when the ligand exchange occurs at surface level (Goldberg and Johnston 2001; Cea et al., 2005). In fact, during this process PCP competes with the anions for the binding sites present on the fungi

walls at an acidic pH level. Mathialagan and Viraraghavan (2009) demonstrated that at a neutral or basal pH value, the fungal biomasses present a net negative charge, therefore, electrostatic repulsion toward the PCP in its anionic form can occur.

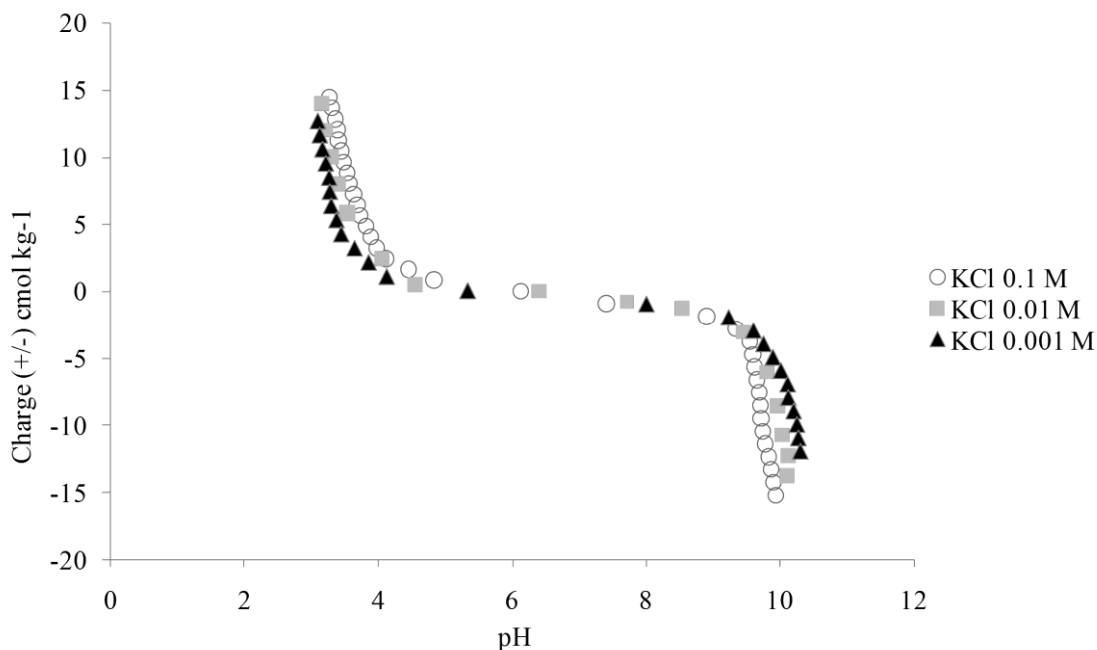


Fig. 1 Potentiometric titration curves of live pellets of *A. discolor* with three KCl electrolyte solutions at 25 ± 1 °C.

On the other hand, at a low pH value the biomass could be protonated and the PCP could be adsorbed by the electrostatic process of the cell wall (Deng et al. 2009; Rubilar et al. 2012) i.e. reducing the pH value, the surfaces of the biomass get hydronium ions which may increase the interaction between PCP and the binding sites of the bio adsorbent. Moreover, the pH levels of the medium influence the ionization state of some functional groups present on the fungal cell wall (Kumar et al. 2009). However, when pH values of the PCP solution are close to its pK_a value (4.75), the adsorption increases due to hydrophobic interactions with the surface in its neutral form (Cea et al. 2005).

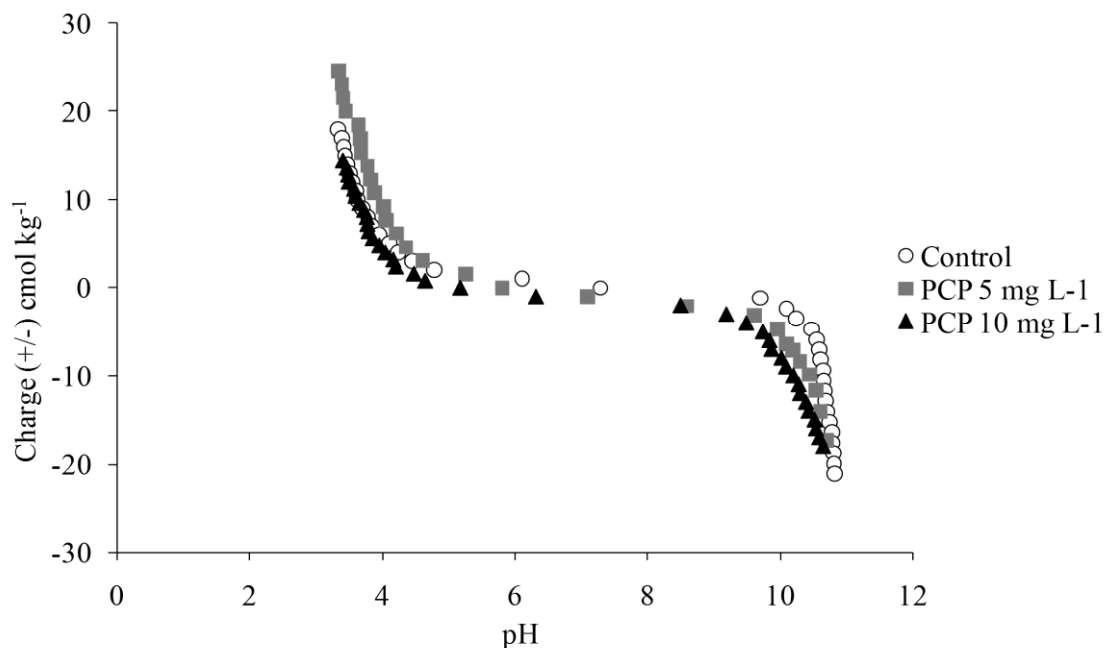


Fig. 2 Potentiometric titration curves of live pellets of *A. discolor* in a KCl 0.1M electrolyte solution at 25 ± 1 °C with different concentrations of PCP.

3.4.3. Effect of PCP concentration and pH levels on the adsorption capacity of *A. discolor*

More than PCP concentrations, the pH levels were the main parameter that affected PCP adsorption for *A. discolor*. In fact PCP adsorption, like other chlorophenols, is strongly influenced by the pH values (Diez et al. 1999; Cea et al. 2005; Kumar et al. 2009; Rubilar et al. 2012). Nevertheless, the pH level was stabilized before inserting the pellets of *A. discolor* into the bottles for the PCP adsorption experiment. After 24 h the pH value was within the range of 5.27 - 5.51 and 5.13 – 6.36 for 5 and 10 mg PCP L⁻¹, respectively (Fig. 3a and 3b). *A. discolor* changed the pH of the solutions in such a way that it was ideal for the growth and vitality (Rubilar et al. 2007; Tortella et al. 2008; Rubilar et al. 2012). The same result was obtained by Jacobsen et al. (1996) in

adsorption and desorption of PCP experiments by way of microbial biomass. After one hour the pH that was measured was decidedly different than that which was stabilized at the start. The pH value is an important parameter for the growth of fungi and our results indicated that the best growth was between a pH of 3.2 and 6.5, with an optimum range being between a pH of 4.5 and 5.5 (Hung 1983).

PCP adsorption of *A. discolor* was $> 80\%$ in the pH range of 5.13 – 5.51 for both of the pollutant compound concentrations tested. No statistical difference was detected at 5 mg PCP L⁻¹ (Fig. 3a). While a significant decrease was observed at 10 mg PCP L⁻¹ in the pH range of 5.81 – 6.36 (Fig. 3b). This decrease in PCP adsorption by dead biomass of *A. discolor* at a pH of > 5.5 was detected also by Rubilar et al. (2012). In fact, in their study, increasing the pH level from 5.0 to 6.0, the percentage of PCP adsorption decreased 37% to the initial PCP concentration of 20 mg L⁻¹. On the other hand increasing the pH from 5.5 to 6.0, the percentage of PCP adsorbed decreased to only 8% of the initial PCP concentration at 20, 30 and 50 mg L⁻¹. Interesting results were obtained by Mathialagan and viraraghavan (2009) in the PCP adsorption/pH-dependent experiments. They detected PCP adsorption activity from aqueous solutions of non-viable *Aspergillus niger* biomass. Various forms of *A. niger* biomass that were chemically altered were tested for their potential in the removal of PCP. It was found that PCP removal was pH dependent; PCP removal decreased with the increase in pH values for all types of biomass, except when the fungus was treated with cetyltrimethylammonium. In this case it showed 100% removal at 1 mg PCP L⁻¹ at all pH levels. For the other forms of *A. niger* biomass that were altered, PCP adsorption was $> 80\%$ at a pH range between 3.0 and 5.0.

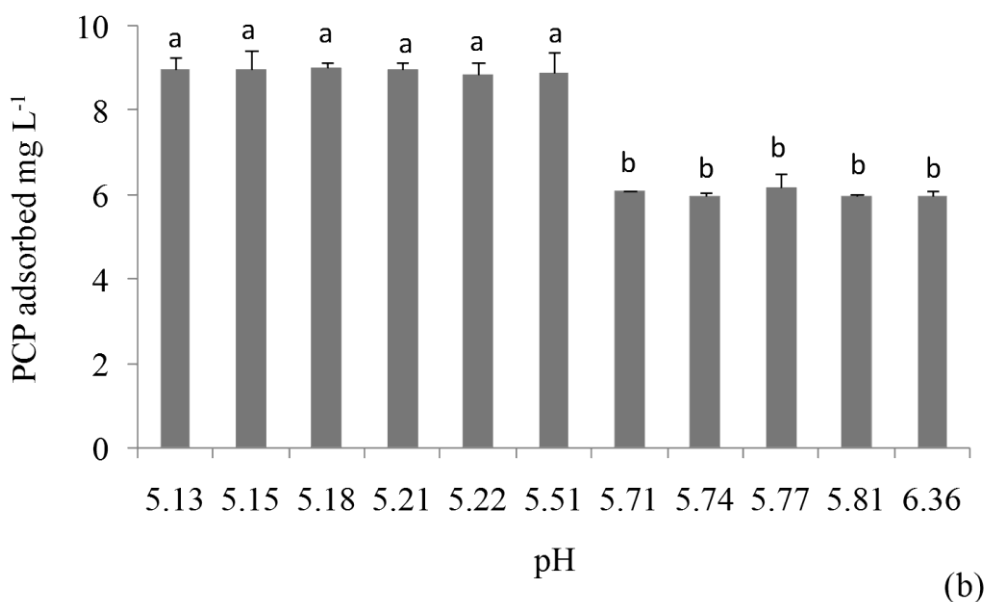
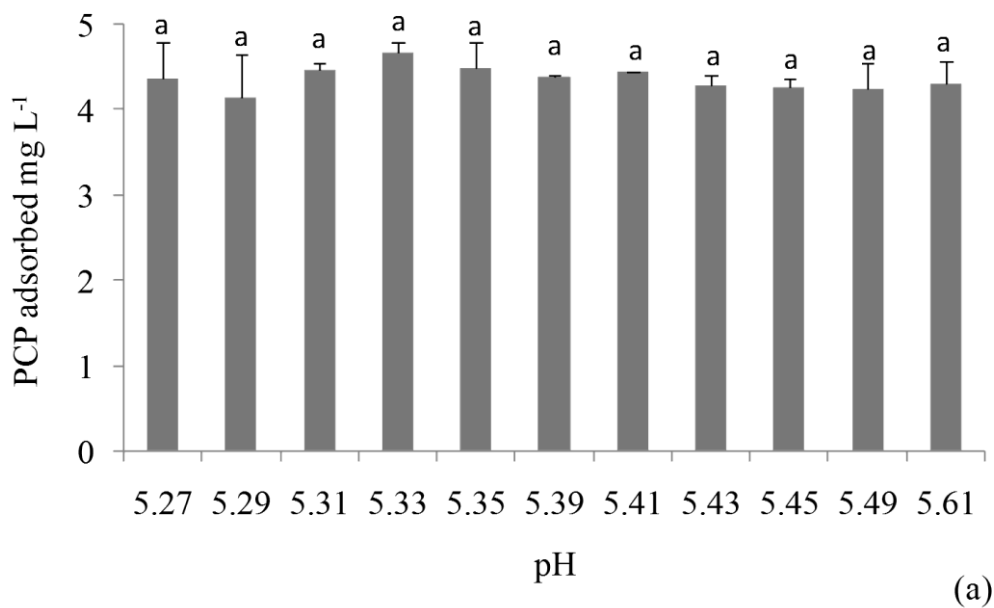


Fig. 3 Effect of pH levels on the adsorption capacity of *A. discolor* pellets (mean \pm standard deviation) at 25 ± 1 °C for 24 hours of (a) 5 mg PCP L⁻¹ (b) 10 mg PCP L⁻¹. Different small letters refer to significant differences ($P < 0.05$) among PCP sorption values.

While the PCP adsorption capacity of the fungus was < 35% at alkaline pH levels. A strain of *Phanerochaete chrysosporium*, killed by heating, adsorbed the highest amount of PCP, about 75 %, at a pH of 6.0 in the experiment conducted by Logan et al. (1994). *Trametes versicolor*, *Ganoderma lobatum* and *Inonotus dryophilus* were able to adsorb 70% of the 80 mg PCP L⁻¹ after 48 h. Brandt et al. (1997) evaluated the influence of the pH levels on adsorption and desorption behavior of PCP by *Mycobacterium chlorophenolicum*. The pH values were found to be an essential parameter which affects the PCP adsorption. The adsorption capacity increased with the decrease of pH values. At a pH of 5.4 the adsorption by bacterium was completely irreversible, while the complete desorption was obtained at a pH of 7.0. At a pH between 6 and 8, Jianlong et al. (2000) obtained better results on adsorption behavior of PCP by microbial biomass in the aqueous solution. Moreover, they discovered that the biomass concentration is an important parameter which affects the PCP adsorption activity which increases with the decreasing biomass concentration. PCP solubility is pH-dependent and its adsorption by fungus or soil is more effective at a lower pH (Cea et al. 2005; Mathialagan and Viraraghavan 2009; Rubilar et al. 2012). Moreover, PCP adsorption was affected by ionic strength, essentially due to the anionic nature of the pollutant compound. Increasing the ionic strength, PCP adsorption increased, presumably thanks to the electrostatic attraction with the microorganism's cell wall.

The pH level of the medium is naturally a fundamental parameter also in adsorption of phenols and others chlorophenols (Aksu 2005; Ahmaruzzaman 2008). In the study done by Rao and Viraraghavan (2002) the maximum adsorption of phenol was obtained at a pH of 5.1 with sulfuric acid pretreated and a non-viable *A. niger* biomass. At a pH of 5.0, Wu and Yu (2006) obtained the highest adsorption capacity, of about 8%, of the 2, 4-dichlorophenol from the aqueous solution of *P. chrysosporium* non-living pellets. Kumar et al. (2009) evaluated the adsorption capacity by changing the pH values of *Trametes versicolor* *polyporus* versus phenol, 2-chlorophenol and 4- chlorophenols.

The results showed that the adsorption by the fungus increased as the pH value was increased from 3.0 to 6.0. The same results were seen by Denizli et al. (2005), who used dried and dead biomass of *Pleurotus sajor-caju*. In fact, the fungus showed higher adsorption capacities dealing with the removal of phenols and chlorophenols compounds from water at a pH of 6.0. In a pH range of 5.0 – 6.0 the bacterium *Bacillus subtilis* adsorbed 80% of 2, 4, 6-trichlorophenol within 3 hours. When the pH solutions were > 7.0 the adsorption capacity lowered to 10% and when the pH level was > 9 it was equal to the 2, 4, 6-trichlorophenol, but adsorbed 0% (Daughney et al. 1998). The invasive macroalga *Sargassum muticum* showed a small capacity, depending on the pH values, to absorb 2-chlorophenol and 4-chlorophenol (Rubin et al. 2006). In the alkaline medium, interesting results were obtained by Navarro et al. (2008) in the adsorption of phenol as a function of the initial pH solution involving marine seaweeds *Lessonia nigrescens* and *Macrocystis integrifolia*. These two organisms showed a higher adsorption rate at pH levels of 8 and 10 with values between 10% and 35%, respectively. Similar results were obtained by Bayramoglu et al. (2009) in the adsorption of phenol and 2-chlorophenol in an aqueous solution while using *Funalia trogii* pellets. The fungus was able to adsorb 75% of 200 mg L⁻¹ of the phenols at a pH of 8.0.

3.4.4. FTIR analysis

The FTIR spectrum of live pellets of *A. discolor* are shown in Fig. 4. This analysis allows us to obtain information on the types and nature of interactions between the fungal pellets and the PCP. As shown in Fig 4, the spectrum before and after PCP adsorption by the *A. discolor* biomass had a similar trend while observing 13 troughs. Therefore, the following discussion on FTIR spectrum on the before and after PCP adsorption by *A. discolor* will be suitable for both of the patterns. The troughs in the FTIR spectrum for pellets before and after PCP adsorption were annotated with an

“A”, “B”, “C”, “D” and so on up to “M”. An intense and primary trough was observed at 3471 cm^{-1} (marked A) which could be indicative of O – H and N – H stretching vibrations. This trough was larger than the others displayed in the two trends. The second and third neighboring troughs (marked B and C) observed at 2962 and 2862 cm^{-1} were indicative of a C – H group vibration of alkane ($-\text{CH}_2$) and methyl ($-\text{CH}_3$) groups. The fourth intense trough at 1656 cm^{-1} (marked D) was due to the C = O stretching vibration mode of the primary and secondary amides (NH_2CO). The fifth and sixth troughs at 1560 and 1460 cm^{-1} (marked E and F) were indicative of the – NH stretching group localized in the cell wall structure of the fungi. The seventh and eighth troughs at 1438 and 1373 cm^{-1} (marked G and H) were due to the C – O stretching vibrations of carboxylic acids derivatives. The ninth troughs at 1249 cm^{-1} (marked I) was due to the C – O stretching vibrations of ketones, aldehydes and lactones. The tenth, eleventh and twelfth troughs (marked J, K and L) at 1164 , 1080 , and 1043 cm^{-1} , respectively, are characteristic adsorption troughs indicating the presence of phosphate functional groups stretching ($\text{P} = \text{O}$ and $\text{P} - \text{O}$) on the fungal biomass. The thirteenth trough was at 557 cm^{-1} (marked M) which could correspond to O – C – O scissoring and C – O bending vibrations. The analysis of the functional groups previously carried out suggests that amides, alkanes, carboxylates, carboxyl and hydroxyl groups could be active in the PCP adsorption by *A. discolor* pellets, as described by Rubilar et al. (2012) and Kumar et al. (2009). On the other hand, amino and phosphate groups, as detected by Mathialagan and Viraraghavan (2009), could have a secondary role or possibly not even play a role in PCP adsorption. The FTIR spectrum trends of before and after PCP adsorption by *A. discolor* were similar, indicating that the fungus was not influenced significantly by the presence of the contaminant. The same results were obtained in the studies produced by Rubilar et al. (2012), and Mathialagan and Viraraghavan (2009).

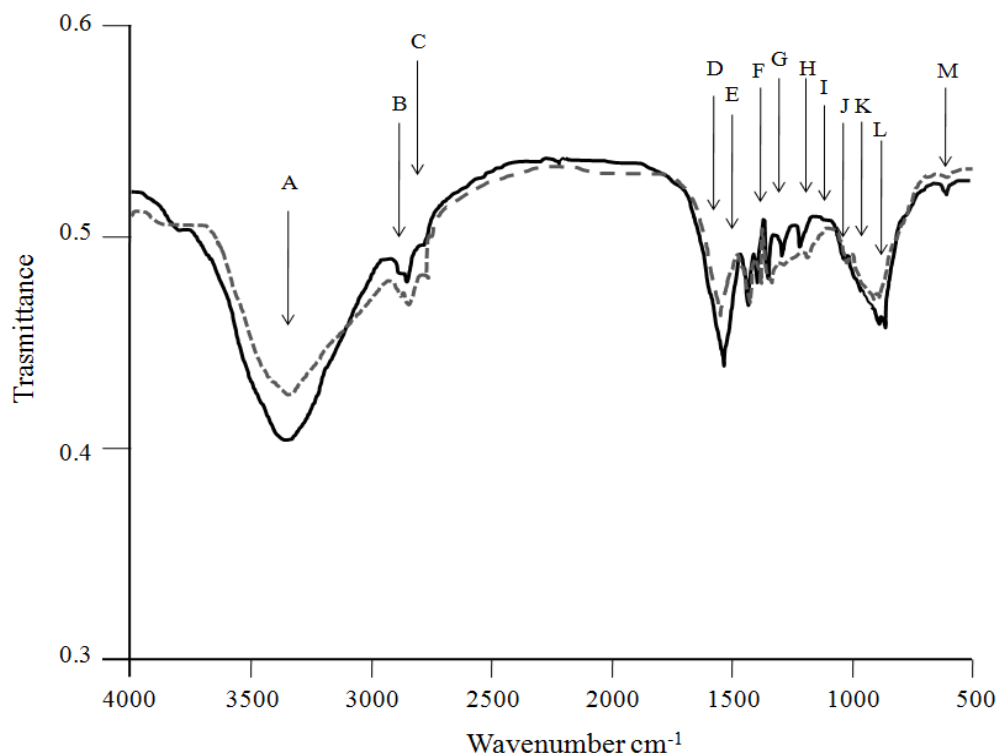


Fig. 4 Spectrum of FTIR analysis of *A. discolor* pellets. Solid black line indicates FTIR of only *A. discolor* biomass while, broken grey line indicates FTIR of *A. discolor* biomass after sorption of 10 mg PCP L^{-1} at 25 ± 1 °C for 24 hours. Capital letter marked significant troughs of functional groups.

Different results, however, were obtained for *Penicillium chrysogenum* (Loukidou et al. 2003), *Funalia trogii* (Bayramoglu et al. 2009) and *Trametes versicolor polyporus* (Kumar et al. 2009) where the pollutant compounds changed the amount and the nature of the biomass functional groups. The analysis of the functional groups previously carried out suggests that amides, alkanes, carboxylates, carboxyl and hydroxyl groups could be active in the PCP adsorption by *A. discolor* pellets, as described by Rubilar et al. (2012) and Kumar et al. (2009). On the other hand, amino and phosphate groups, as detected by Mathialagan and Viraraghavan (2009), could have a secondary role or possibly not even play a role in PCP adsorption. The FTIR spectrum trends of before and after PCP adsorption by *A. discolor* were similar, indicating that the fungus was not

influenced significantly by the presence of the contaminant. The same results were obtained in the studies produced by Rubilar et al. (2012), and Mathialagan and Viraraghavan (2009). Different results, however, were obtained for *Penicillium chrysogenum* (Loukidou et al. 2003), *Funalia trogii* (Bayramoglu et al. 2009) and *Trametes versicolor polyporus* (Kumar et al. 2009) where the pollutant compounds changed the amount and the nature of the biomass functional groups.

3.4.5. Assessing *A. discolor* growth and Remazol Brilliant Blue R decolourization

After 5 days of incubation no significant difference was highlighted in the mycelium growth among the *A. discolor* pellets PCP adsorption (5 or 10 mg L⁻¹) and without the PCP adsorption (control). All the fungal pellets grew 1.5 ± 0.2 cm day⁻¹ showing that the PCP adsorption after 24 hours at 5 or 10 mg L⁻¹ did not influence the hyphal growth in relation to the control pellet (without PCP). This effect could be due to the low PCP concentrations used. In this sense, Tortella et al. (2008) showed that mycelium growth of *A. discolor* was reduced by 70%, 60% and 100% at 50 mg PCP L⁻¹, 2,4-dichlorophenol and 2,4,6-Trichlorophenol, respectively, in the tolerance and degradation experiments.

Further, the Remazol Brilliant Blue R was completely decolorized by live pellets of *A. discolor* (control and PCP adsorption at 5 and 10 mg L⁻¹) in only 5 days. In all cases, the fungal growth and decolorization were positively correlated i.e. the diameter of 85 mm was detected for the colony size and decolorized zone. *A. discolor* pellets showed a rapid capacity of growth and to decolorize the Remazol Brilliant Blue R, obtaining a better performance than that of genera *Agrocybe*, *Auricularia*, *Ganoderma*, *Phellinus*, *Pleurotus*, *Psilocybe*, *Lentinus* and *Trametes* which were tested by Machado et al.

(2005) and versus the species *Dichomitus squalens* (Eichlerová et al. 2006) and *Bjerkandera adusta* (Eichlerová et al. 2007).

3.5. Conclusions

In this study the adsorption of PCP by *A. discolor* pellets in amber bottles was pH-dependent and was higher at a pH of 5 – 5.5, all the while significantly decreasing by < 60% at 10 mg PCP L⁻¹ in the pH range of 5.71 – 6.41. The fungal growth and Reason Brilliant Blue R decolorization was not affected by the PCP adsorption after 24 h. The live pellets may also be used as a natural absorbent for PCP. The FTIR results showed that amides, alkanes, carboxylates, carboxyl and hydroxyl groups can be important functional groups to the adsorption of PCP by fungus. Finally, the live pellets of *A. discolor* may be considered a good biosorbent for liquid solutions contaminated by PCP.

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Trametes versicolor

4. Assessing the effectiveness of *Byssochlamys nivea* and *Scopulariopsis brumptii* in pentachlorophenol removal and biological control of plant pathogens

4.1. Abstract

Two areas in fungi research capable of producing beneficial effects on the economy and environment are bioremediation and biological control. Two fungal strains were isolated from an artificially PCP-contaminated soil during a long-term experiment and identified as: *Byssochlamys nivea* and *Scopulariopsis brumptii*. The fungal strains were tested in sensitivity and degradation to pentachlorophenol (PCP) and as antagonist against Oomycetes *Phytophthora cinnamomi* and *Phytophthora cambivora*. The fungal strains showed a good PCP tolerance (12.5 and 25 mg PCP L⁻¹) although the hyphal size, biomass, patulin and spore production decreased with increasing PCP. It was shown that these two fungi can completely deplete 12.5 and 25 mg PCP L⁻¹ in submerged culture after 28 days of incubation at 28 °C. Electrolyte Leakage Assays showed that the fungi have a good tolerance at 25 mg PCP L⁻¹ and do not produce toxic compounds for the plant. *B. nivea* and *S. brumptii* were able to inhibit the growth of *P. cinnamomi* and *P. cambivora* in solid and liquid culture. Volatile organic compounds (VOCs) did not produce growth reduction of oomycetes strains. These results indicate that *B. nivea* and *S. brumptii* have an interesting potential in bioremediation and biocontrol strategy.

4.2. Introduction

The use of beneficial fungi and bacteria for bioremediation and biological control (biocontrol) of plant diseases has made tremendous strides in numerous biotechnology applications. In recent years the need to find a global approach to environmental and

agricultural issues has set out the challenge to discover microorganisms useful for both bioremediation and biocontrol research (Griffin 2014). In this way, the contribution to soil, water and crop protection will be twofold and achieved through the use of low-cost biotechnologies promoting ecological alternatives. This approach is primarily focused on how to use microorganisms strains to inhibit the dangerous advance of plant pathogens and to deplete environmental contaminants (Sylvia et al. 2005; Singh et al. 2011). The fungi that play important roles in biocontrol and bioremediation strategy are numerous; some of these are grouped as mycorrhizal and endophytic fungi (Jeffries et al. 2003; Griffin 2014). The endophytic fungus *Phomopsis* sp. was able to use the 4-hydroxybenzoic acid as only carbon source (Chen et al. 2011) and to inhibit the cerambycidae *Physocnemum brevilineum* which is a vector of the elm pathogens, such as *Ceratocystis ulmi* (Webber 1981). The fungi of *Glomus* genus are arbuscular mycorrhizas used in biocontrol of soil-borne plant pathogens (Azcón-Aguilar and Barea 1996) and as heavy metal biosorbent in the soil (Leyval et al. 2002). Filamentous fungal species belonging to the genus *Trichoderma* are able to counteract some plant pathogens by means of mycoparasitism and antibiosis (Howell 2003; Lorito et al. 2010) and, simultaneously, to deplete pollutants including chlorophenols, heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Tripathi et al. 2013). *Aspergillus flavus* was used as biosorbent of heavy metals (Deepa et al. 2006), phenol degraders (Ghanem et al. 2009) and in promoting *Phytophthorae*'s growth inhibition (Evidente et al. 2006). *Penicillium* spp. has demonstrated excellent ability to degrade different xenobiotic compounds such as phenolic compounds, PAHs and heavy metals (Leitão 2009). Furthermore some strains of *Penicillium* genus were used as biocontrol, such as *Penicillium funiculosum* and *Penicillium janthinellum* that were able to limit the *Phytophthorae* root rots of azalea (Ownley and Benson 1992; Fang and Tsao 1995). Some species of *Verticillium* genus were able to remove petroleum products and PAHs in soil (Gadd 2001; Singh et al. 2011) and to control numerous plant pathogens such as

fungi, bacteria and nematodes (Mérillon and Ramawat 2012). Other interesting ecological groups are for example the white-rot fungi. *Trametes versicolor* has showed promising biocontrol activities against *Fusarium oxysporum* (Ruiz-Dueñas and Martínez 1996) and represents one of the most important organism used in bioremediation study against numerous contaminants (Gadd 2001; Singh 2006; Bosso et al. 2011).

In the present study we carried out with *Byssoschlamys nivea* (Westling, 1909) and *Scopulariopsis brumptii* (Salvanet-Duval, 1935) *in vitro* experiments to reach the following goals:

1. remove highly chlorinated compound extensively used as a biocide in wood industry and persistent environmental contaminant of soil and water: the pentachlorophenol (PCP).
2. control *Phytophthora cinnamomi* (Rands, 1922) and *Phytophthora cambivora* (Buisman, 1927); two plant pathogen that cause “ink disease”, one of the most destructive diseases affecting European Chestnut trees (*Castanea sativa* Miller).

4.3. Materials and Methods

4.3.1. Isolation of fungal strains

Two fungal strains were isolated from an artificially PCP-contaminated soil during a long-term experiment (Scelza et al. 2008) according to a slightly modified protocol of Martin (1950). Briefly, 1gr of soil sample was suspended in 100ml of distilled sterile water to make microbial suspensions (10^{-1} to 10^{-7}). Dilutions between 10^{-1} and 10^{-5} were used to isolate fungi. An amount of 1 ml of microbial suspension of each concentration was added to sterile Petri dishes containing 20 ml of potato dextrose agar

(PDA) (5 g L⁻¹ potato; 20 g L⁻¹ dextrose; 15 g L⁻¹ agar). One percent of streptomycin solution or 1 ml of lactic acid (diluted to 20%) was added to the medium to prevent bacterial growth. The Petri dishes were then incubated at 28 °C in the dark and were observed everyday up to 5 days. After isolation, the fungi were transferred into PDA slant cultures tubes and stored at 4 °C.

4.3.2. DNA extraction, PCR amplification and analysis

The fungi were transferred from slant cultures tubes to PDA plates and incubated at 25 °C for 7 days. The procedure described later was carried out for each fungus. Two plugs were taken from fungal plates and placed in flasks with potato dextrose broth (PDB, SIGMA) and incubated for 30 days at 25 °C on orbital shaker (120 r.p.m.). Mycelium harvested from the flasks was dried, frozen, lyophilized and stored into falcon tubes. DNA extraction was realized following the method of de Graaff et al. (1988). Genetic analysis of Ribosomal DNA was determined by PCR of the internal transcribed spacer (ITS) sequences. We used a PCR based approach to amplify, using primers ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS2 (5'- GCTGCGTTCTTCATCGATGC -3'), fungal DNA fragments. The following parameters were used in thermocycler amplification: 1 minute initial denaturation at 94 °C, followed by 30 cycles of 1 minute denaturation at 94 °C, 1 minute primer annealing at 50 °C, 90 seconds for extension at 72 °C and final extension period of 7 minutes at 72 °C. The PCR product was gel electrophoresed and quantified by Qubit® 2.0 Fluorometer. Quantified PCR product was sent to Eurofins MWG Operon to sequencing. Finally, DNA sequence was aligned using Basic Local Alignment Search Tool (BLAST) that finds regions of local similarity between sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). After molecular identification the fungi cultures were also observed on the basis of macroscopic and microscopic characteristics.

4.3.3. Chemicals

PCP (>99% purity) and HPLC solvent were obtained from Sigma Aldrich (Germany). All other chemicals reagents were purchased by BD (United State of America).

4.3.4. Sensitivity test to PCP in plate culture

Sensitivity to PCP by fungi was evaluated in Petri dishes. The fungal strains were grown on PDA at different PCP concentration (12.5 and 25 mg L⁻¹). Controls were cultured without PCP. All samples were incubated at 25 °C for 7 days. Tolerance to PCP was determined by measuring the diameter of the colony (Tomassini et al. 2001), hyphal size (this was easily achieved measuring with a microscope the hyphal thickness in µm) and spore production (St-Arnaud et al. 1996). To determine hyphal thickness and spore production 100 measurements were carried out for each replicate.

4.3.5. Sensitivity test to PCP and PCP removal in submerged culture

Sensitivity to PCP by fungi was determined also in submerged culture. Two plugs of 8 mm diameter of active mycelia taken from PDA cultures of 7 days old were grown in potato dextrose broth (PDB) (5 g L⁻¹ potato; 20 g L⁻¹ dextrose) and PCP at 12.5 and 25 mg L⁻¹. Controls were cultured without PCP. Cultures were grown in Erlenmeyer flask of 250 ml containing 100 ml of medium. All samples were incubated at 28 °C and shaken at 125 rev min⁻¹ for 28 days. Sensitivity test was evaluated measuring the dry weight biomass. The biomass was separated from PDB by filtration using Whatman Filter MN 640 d - 110 mm diameter (Macherey-Nagel). The biomass was washed with distilled water, dried in an oven at 50 °C for 24 h and weighed. PCP adsorption and degradation analysis were carried out in submerged culture using the same medium, PCP concentration and fungi inoculation and incubation mode as previously described.

The analytical methods used to determine the PCP in liquid culture and biomass were extensively described in Tomassini et al. (2001).

4.3.6. Utilization of PCP as sole carbon source

The fungal strains were tested for their ability to utilize PCP in liquid mineral medium with PCP as sole carbon source. The fungi were inoculated into a flask containing 12.5 and 25 mg PCP L⁻¹ in a medium with the following composition (g L⁻¹): KH₂PO₄ (2.0); MgSO₄ · 7H₂O (0.5); CaCl₂ (0.1); NH₄Cl (0.12); ZnSO₄ · 7H₂O (0.1); MnCl₂ · 4H₂O (0.3). The control was obtained with the same mineral medium adding 10 g L⁻¹ of glucose as carbon source. The pH of the medium was adjusted to 5.5 before sterilization. The flasks were inoculated with two plugs of 8 mm diameter of active mycelia taken from PDA cultures of 7 daysold. . This procedure was done for each fungi individually. The incubation was carried out at 28 °C on orbital shaker incubator at 120 r.p.m in the dark for 28 days. The use of PCP as sole carbon source was evaluated measuring the dry weight biomass as described above. The analytical methods used to determine PCP in liquid mineral medium were extensively described in Tomassini et al. (2001).

4.3.7. Influence of PCP on production of patulin in submerged culture

From the submerged culture used for PCP removal experiment (described above) were collected 10 ml of sample and analyzed for patulin production. In a 50ml falcon were inserted 10 ml of sample, 15gr of Na₂SO₄ (Sigma-Aldrich), 2 gr of NaHCO₃ (Fluka) and 10 ml of a mixture 60+40 [v/v] composed by C₄H₈O₂ (Clean Consult)/ C₆H₁₄ (Carlo Erba) and mechanically mixed for 4 minutes. After which the falcon was centrifuged at 2000 r.p.m. for 1 minute. From the falcon 2.5 ml of sample was collected and washed in a HLPC C18 column (Phenomenex) with 3 ml mixture of ethyl acetate/hexane. The eluate was collected in a little tube containing 50 µl of

C₂H₄O₂ (Merck), resuspended in 1 ml of water with pH 4 and transferred into a vial. The determination of the patulin was carried out by HPLC (Shimadzu) equipped with autosampler SIL-20A, two pumps LC-20AD and a UV / VIS SPD-20A detector; wavelength: 276 nm. The column used was Gemini 5 μ C18 110 Å (150 x 2 mm) (Phenomenex). Mobile phase A (95%): acetic acid with 1% of distilled water. Phase B: CH₄O (Carlo Erba). Flow rate: 1ml / min. Under these conditions the retention time of patulin was around 14 minutes and the LOD equal to 5 ppb.

4.3.8. Electrolyte Leakage Assay (ELA)

ELA was carried out on our fungi to assess: i) PCP toxic effect on mycelium; ii) the potential damage on plant cell wall and membrane of fungal metabolites. This second experiment was conducted to assess the potential use of bioremediation strategy in field studying.

For the first experiment, 6 plugs of 5 mm diameter of active mycelia were taken from water agar cultures of 7 days old (WA, Carlo Erba) and incubated in the dark for 24 h in beakers containing distilled water + 25 mg PCP L⁻¹. The control was obtained incubating the fungus with only distilled water. After the incubation all fungi plugs were washed and transferred into a becker containing 15 ml of distilled water. The measurements were conducted after 1, 2, 4, 7 and 8 hours except for the first and last analysis that were made after 0.30 and 24 hours, respectively.

For the second experiment, 10 tomato stem pieces (5-mm long) were incubated overnight in culture filtrate of the fungi and only in PDB (control). Fungal culture filtrate was obtained from PDB medium with active mycelia cultured for 7 days. The culture filtrate was separated from the mycelium by filtration with Whatman Filter MN 640 d - 110 mm diameter (Macherey-Nagel). After the incubation all the stem pieces were washed and transferred into a becker containing 15 ml of distilled water. The

measurements were set hourly except for the first and last analysis that were made after 0.30 and 24 hours, respectively.

In all the experiments the corresponding conductance was measured in microsiemens using a conductivity-meter with 20 electrodes (range 20–200 mS/cm; K¹/41). The conductivity values were calculated as the difference from the reading at the beginning of the assay (Evidente et al. 2009).

4.3.9. Antagonistic assays against *Phytophthora* spp. in vitro

The antagonistic assays were carried out among our fungi against two plant pathogens: *Phytophthora cambivora* and *Phytophthora cinnamomi*. These two Chromisti are stored into PDA slant tubes at 18 °C in the laboratories of Forest Pathology of the Department of Agriculture (University of Naples Federico II, Italy). Colony interaction and competition was analyzed in dual culture method (Chand and Logan 1984) in Petri plate (85 mm diameter) containing 20 ml of PDA. The diameter of the colony was measured. The volatile organic compounds (VOC) were analyzed following the method described by Dennis & Webster (1971) measuring the radial extension of the colonies. The biomass weight was detected in flasks containing 100 ml of PDB. The biomass was separated from the PDB by filtration using Whatman Filter MN 640 d - 110 mm diameter (Macherey-Nagel). After which, the mycelium was washed with distilled water, dried in an oven at 50 °C for 24 h and weighed. In all experiments an antagonist plug of 5mm diameter was added 24 h before the *Phytophthora* plug. The Petri plate and flasks were incubated at 25°C in the dark for 10 days. Controls were carried out placing in Petri dishes and flask a plug of pathogen and only PDA.

4.3.10. Statistical analysis

All statistics analysis were carried out using XLSTAT version 2013.1. Analyses of variance (ANOVA) followed by a least significant difference (LSD) test at $P < 0.05$ was used to determine significant differences among means. All experiments were performed in triplicate.

4.4. Results

4.4.1. Fungal strains identification

The fungal strains were identified as: *Byssoschlamys nivea* (Westling, 1909) (100% of identity - Accession code: AF360391.1) *Scopulariopsis brumptii* (Salvanet-Duval, 1935) (98% of identity - Accession code: HG380457.1). These results were confirmed also by macroscopic and microscopic characteristics.

4.4.2. Sensitivity test to PCP

In experiments performed with PDA Petri dishes, the fungal radial growth and hyphal thickness showed a significant decreased at 25 mg PCP L⁻¹ only for *B. nivea*. The fungal growth rate of *B. nivea* considerably decreased at increasing PCP concentration. Instead, *S. brumptii* suffered a reduction of growth rate when the PCP concentration was \geq of 12.5 mg L⁻¹ (Table 5). The spore production decreased when PCP concentration was 25 mg L⁻¹ for both fungi. The biomass production, evaluated in liquid culture, suffered a significant decrease when PCP was \geq of 12.5 mg L⁻¹ for both fungi. The fungi did not grow when PCP was used as sole carbon source in liquid mineral medium (Table 5). Always determined in flasks, the patulin productions significantly decreased at increasing PCP concentration for both fungi (Table 5).

Table 5. Sensitivity test to PCP by fresh fungal mycelium at different PCP concentrations. Different small letters refer to significant differences ($P < 0.05$) between PCP sensitivity of *B. nivea* and *S. brumptii* at the same PCP concentration. Different capital letters refer to significant differences ($P < 0.05$) among PCP sensitivity of *B. nivea* and *S. brumptii* at different PCP concentration.

	Control		12.5 mg PCP L ⁻¹		25 mg PCP L ⁻¹	
	<i>B. nivea</i>	<i>S. brumptii</i>	<i>B. nivea</i>	<i>S. brumptii</i>	<i>B. nivea</i>	<i>S. brumptii</i>
Radial mycelium extension (mm)	85 ^{aA} ± 0	85 ^{aA} ± 0	83.1 ^{aA} ± 2.4	85 ^{aA} ± 0	64.8 ^{aB} ± 17.5	83.3 ^{bA} ± 2.5
Growth rate daily (mm day ⁻¹)	10.6 ^{aA} ± 0	10.5 ^{aA} ± 0.1	7.5 ^{aB} ± 0.2	7.7 ^{aB} ± 0.1	5.8 ^{aC} ± 1.5	7.5 ^{bB} ± 0.2
Hyphal size (µm)	5.5 ^{aA} ± 0.2	7.2 ^{bA} ± 1.2	5.3 ^{aA} ± 0.3	7.2 ^{bA} ± 0.9	3.2 ^{aB} ± 0.9	7.2 ^{bA} ± 0.7
Production of Spore (x 1250000)	666.5 ^{aA} ± 7.8	225.5 ^{bA} ± 0.7	640.5 ^{aA} ± 13.4	126.5 ^{bB} ± 2.1	58.1 ^{aB} ± 4.2	43.3 ^{bC} ± 1.4
Biomass (mg)	184.4 ^{aA} ± 7	218.7 ^{bA} ± 2	51.95 ^{aB} ± 9	106.33 ^{bB} ± 8	50.27 ^{aB} ± 5	27.7 ^{bC} ± 7
Biomass (mg) with PCP as sole carbon source	94.0 ^{aA} ± 1.2	95.3 ^{bA} ± 0.2	0 ^{aB} ± 0	0 ^{aB} ± 0	0 ^{aB} ± 0	0 ^{aB} ± 0
Production of Patulin (mg L ⁻¹)	15.9 ^{aA} ± 0.1	12.4 ^{bA} ± 0.2	10.2 ^{aB} ± 0.2	8.3 ^{bB} ± 0.1	6.1 ^{aC} ± 0.1	4.3 ^{aC} ± 0.1

Table 6. Adsorption and degradation of PCP (12.5 and 25 mg L⁻¹) in 100 ml of PDB by fresh fungal mycelium at different PCP concentrations. Different small letters refer to significant differences ($P < 0.05$) between removal PCP values of *B. nivea* and *S. brumptii* at the same PCP concentration. Different capital letters refer to significant differences ($P < 0.05$) among removal PCP values of *B. nivea* and *S. brumptii* at different PCP concentration.

	Control		1.25 mg PCP L ⁻¹		2.5 mg PCP L ⁻¹	
	<i>B. nivea</i>	<i>S. brumptii</i>	<i>B. nivea</i>	<i>S. brumptii</i>	<i>B. nivea</i>	<i>S. brumptii</i>
PCP adsorption (mg)	-	-	0.03 ^{aA} ± 0.02	0.06 ^{aA} ± 0.02	0.11 ^{aB} ± 0.04	0.08 ^{aA} ± 0.02
PCP degradation (mg)	0.01 ^a ± 0.01	0.03 ^a ± 0.01	1.21 ^{aA} ± 0.03	1.18 ^{aA} ± 0.02	2.38 ^{aB} ± 0.04	2.41 ^{aB} ± 0.02

Table 7. Antagonistic assays of *B. nivea* and *S. brumptii* against *Phytophthora* spp. *in vitro*. Different small letters refer to significant differences ($P < 0.05$) between growth values of *P. cambivora* or *P. cinnamomi* versus the same conditions of antagonism. Different capital letters refer to significant differences ($P < 0.05$) among growth values of *P. cambivora* or *P. cinnamomi* versus different conditions of antagonism.

	Control		<i>B. nivea</i>		<i>S. brumptii</i>	
	<i>P. cambivora</i>	<i>P. cinnamomi</i>	<i>P. cambivora</i>	<i>P. cinnamomi</i>	<i>P. cambivora</i>	<i>P. cinnamomi</i>
Colony interaction (mm)	42.5 ^{aa} ± 0.5	40.1 ^{ba} ± 0.2	12.3 ^{ab} ± 0.2	12.8 ^{ab} ± 0.5	24.2 ^{ac} ± 0.6	23.2 ^{ac} ± 0.8
Volatile organic compounds (mm)	42.5 ^{aa} ± 0.5	40.1 ^{ba} ± 0.2	41.3 ^{aa} ± 0.8	40.1 ^{ba} ± 0.6	41.3 ^{aa} ± 0.8	40.3 ^{aa} ± 0.6
Biomass (mg)	8.7 ^{aa} ± 0.5	8.2 ^{aa} ± 0.5	2.9 ^{ab} ± 0.5	3.0 ^{ab} ± 0.2	5.2 ^{ac} ± 0.5	5.6 ^{ac} ± 0.3

4.4.3. PCP removal capacity

PCP removal ability of *B. nivea* and *S. brumptii* was evaluated in flask containing PDB after 28 days. The trend of PCP adsorption and degradation showed no significant difference between the fungi (Tab. 6). *B. nivea* was able to adsorb the 2.8 and 4.6% of 12.5 and 25 mg PCP L⁻¹. While, at the same concentrations, *S. brumptii* adsorbed the 5.2 and 3.3%. The degradation of PCP was > 95% at any concentration of the contaminant analyzed for both fungi (Table 6). In the control, without fungal mycelium, the PCP removed was < 1%.

4.4.4. Electrolyte Leakage Assay

Two ELA experiments were carried out for *B. nivea* and *S. brumptii* to assess: i) PCP toxic effect on mycelium; ii) the effects of fungal metabolites on plants.

In the first experiment, the incubation with PCP did not produce a significant variation of electrolyte leakage for *B. nivea* in 24 hours. Instead, at the same time, *S. brumptii* showed a decrease of 15% of electrolyte leakage respect to the control incubated only in distilled water (Fig. 5). The increments of conductance were stable after 7 hours for all samples.

In the second experiment, the electrolyte leakage of tomato stem pieces incubated only in PDB and the fungal filtrate culture showed no significant difference after 0.3 and 24 hours. The conductance measured for *B. nivea* during the first eight hours highlighted an increase of electrolyte leakage of the tomato stem. Even in this case the increments of conductance were stable after 7 hours for all samples (Fig. 6).

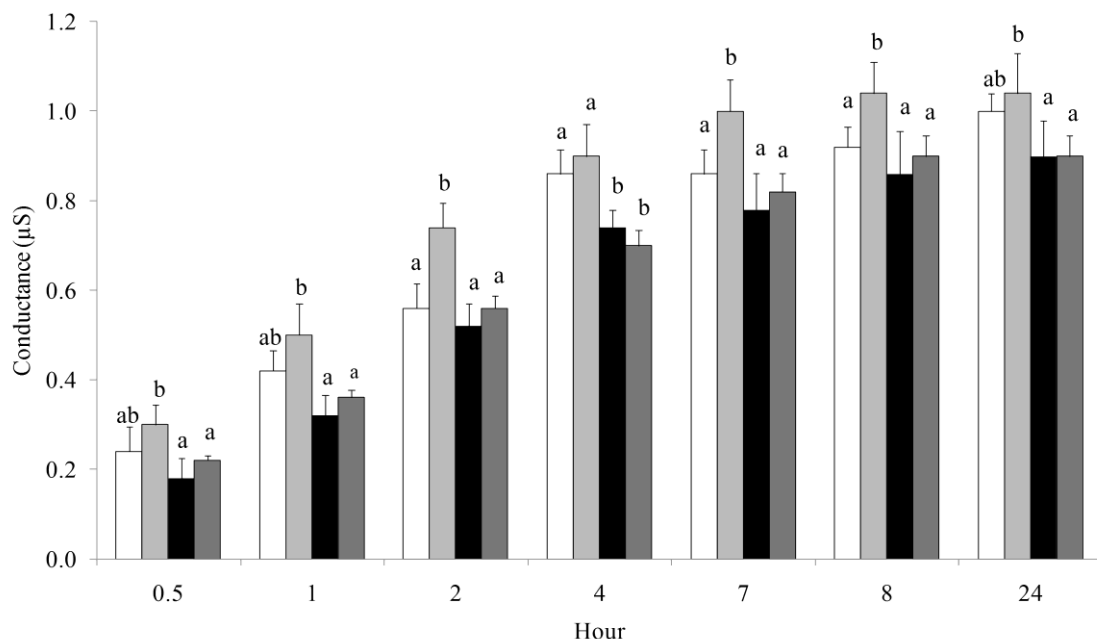


Fig. 5 Toxic effects of 25 mg PCP L⁻¹ on 6 plugs of *B. nivea* and *S. brumptii* after 24 h of incubation using Electrolyte Leakage Assay (ELA). Results are expressed as mean \pm standard deviation. Different small letters refer to significant differences ($P < 0.05$) among PCP sensitivity of: *B. nivea* incubated in distilled water (white bar); *S. brumptii* incubated in distilled water (light grey bar); *B. nivea* incubated at 25 mg PCP L⁻¹ (black bar); *S. brumptii* incubated at 25 mg PCP L⁻¹ (dark grey bar).

4.4.5. Antagonistic assays against *Phytophthora* spp. in vitro

Radial mycelium extension was measured with PDA in colony interactions and VOC experiments. In the first case, the growth of *P. cambivora* and *P. cinnamomi* was reduced to 40 and 60% when these pathogens were cultured with *S. brumptii* and *B. nivea*, respectively (Table 7). The radial growth of *Phytophthora* isolates, measured in VOC experiments in presence of the fungi, showed no significant difference respect to the control (Table 7). Finally, *S. brumptii* and *B. nivea* reduced to 50 and 60% the biomass production of *P. cambivora* and *P. cinnamomi* in flask (Table 7).

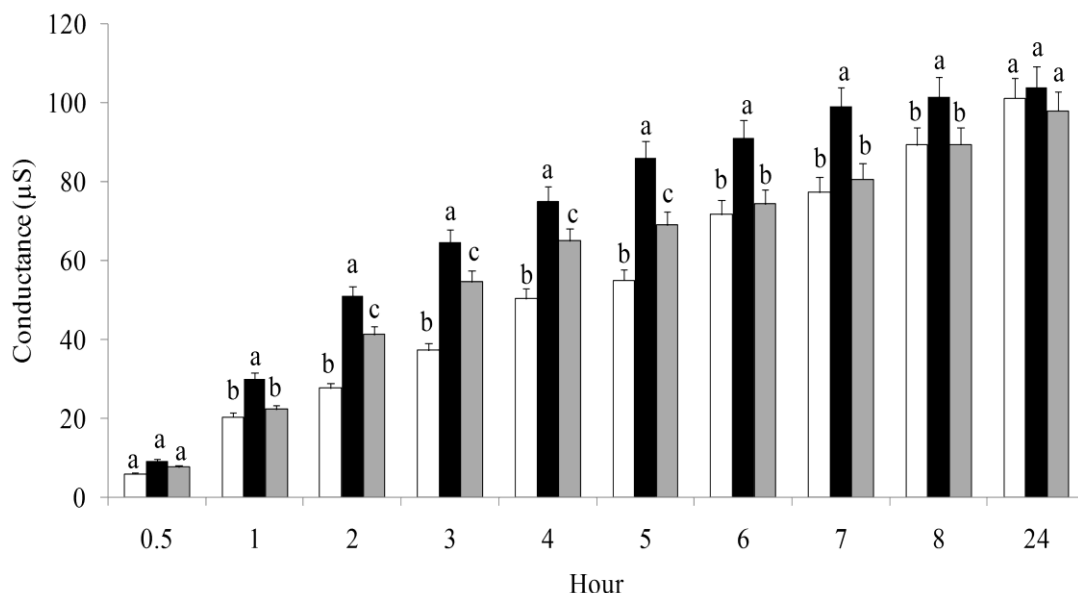


Fig. 6 Toxic effects of *B. nivea* and *S. brumptii* culture filtrates on 10 tomato stem pieces after 24 h of incubation using Electrolyte Leakage Assay (ELA). Results are expressed as mean \pm standard deviation. Different small letters refer to significant differences ($P < 0.05$) among toxicity on tomato stem incubated in: only PDB (control) (white bar); *B. nivea* culture filtrates (black bar); *S. brumptii* culture filtrates (grey bar).

4.5. Discussion

4.5.1. *B. nivea* and *S. brumptii* in PCP sensitivity

The fungal strains showed a good PCP tolerance at the concentrations tested. In Petri dishes, *B. nivea* suffered a decrease of radial mycelium extension, hyphal thickness and daily growth rate when tested at 25 mg PCP L⁻¹. Instead, *S. brumptii* grew without significant limitations due to PCP. Tomassini et al. (2001) showed that an isolate of *Rhizopus nigricans* was able to grow even at 100 mg PCP L⁻¹ while, no growth was detected at 250 mg PCP L⁻¹. Moreover *R. nigricans*' daily growth rate was of 9.8 and

9.6 mm day⁻¹ with 12.5 and 25 mg PCP L⁻¹. Our fungi were slower in growth (with an average of 7 mm day⁻¹ for both fungi) than *R. nigricans*, but, after 5 days, the radial mycelium extension of our fungi was of 55 mm compared to 45 mm of *R. nigricans*. Bomar and Bomar (1999) have analyzed the PCP tolerance of *Aspergillus niger* on PDA. The radial growth of *A. niger* was completely inhibited at 40 mg PCP L⁻¹, while at concentrations of 20 and 10 mg PCP L⁻¹, the mycelium growth was reduced to 88 and 75%, respectively. At the same concentration *A. niger* formed only vegetative structures without conidia formation. *B. nivea* and *S. brumptii* did not suffer of the same radial extension reduction, although, the spore production decreased at increasing PCP concentration. After the experiment the conidia were still able to germinate for both fungi. *Phanerochaete chrysosporium* showed a radial growth rate of 10.3 cm day⁻¹ at 12.5 mg PCP L⁻¹, but its growth rate decreased at increasing PCP concentration, with 8.2 cm day⁻¹ at 25 mg PCP L⁻¹ Tomassini et al. (1996). Tortella et al. (2008) tested 11 white-rot Chilean isolates in resistance and mycelium radial extension at 25 mg PCP L⁻¹ in N-limited solid media. When the fungi were exposed to PCP, only *Lenzites betulina* had a growth rate of 10 mm day⁻¹. Walter et al. (2003) reported that the most tolerant strains at 20 mg PCP L⁻¹ was *T. versicolor* (HR 160) that grew 28 mm day⁻¹.

Exactly like the radial growth, also patulin and biomass production were negatively influenced at increasing PCP concentration. In literature there are no studies on the interaction between patulin and PCP. *B. nivea* and *S. brumptii* reduced to 60% the patulin production at 25 mg PCP L⁻¹. Same results were obtained when the fungi were grown in PDB to evaluate the submerged biomass. In liquid batch cultures Chiu et al. (1998) detected that when some strain were cultured for 7 days with 25 mg PCP⁻¹, this caused reduction in growth of the biomass by 74% in *Volvariella volvacea*; 77% in *Armillaria gallica*; 30% in *Armillaria mellea*; 28% in *Ganoderma lucidum* and 17% in *Pleurotus pulmonarius*. Marcial et al. (2006) evaluated the effects of higher initial PCP concentrations (12.5 and 25 mg L⁻¹) on *Amylomyces rouxii*'s biomass growth. In

cultures with with 25 and 12.5 mg PCP L⁻¹, the biomass growth of *A. rouxii* was of 8 mg after 96 h. When *B. nivea* and *S. brumptii* were cultured in liquid mineral medium with PCP as sole carbon source, the fungi showed no biomass production. Instead when in the flask glucose was added, fungal biomass was achieved for both fungi. This essentially happens because unlike bacteria, fungi do not utilize PCP as a source of carbon and energy. In fungi the degradation of PCP is not consequence of enzyme tools able to do this function but an effect of co-metabolism (McAllister et al. 1996;Field and Sierra-Alvarez, 2008).

The effects of PCP on hyphal size, mycelium morphology and spore production of *B. nivea* and *S. brumptii* were evident at increasing PCP concentration. Although, the PCP never reduced to zero the fungi growth. ELA assays showed that *B. nivea*, after 24 h of incubation at 25 mg PCP L⁻¹, did not suffer a decrease in electrolyte leakage compared to control incubated in distilled water. On the other hand, *S. brumptii* reduced to only 15% the conductance values in 24 h. After 24 h (data not showed) there was no significant difference versus the control. It is widely known that PCP can negatively influence fungi cellular processes, morphology, lipid membrane components, biomass growth, enzymatic activity, sporulation and reproduction capacity (Watanabe 1978; Bajpaia and Banerjib 1992) but in our case, *B. nivea* and *S. brumptii* seem to have a good tolerance to PCP. Tolerance to PCP is the first goal to reach if we want to select specific and effective microorganisms useful in PCP degradation.

4.5.2. *B. nivea* and *S. brumptii* in PCP degradation

Fresh mycelium of *B. nivea* and *S. brumptii* was found to be able to adsorb and degrade PCP. For both fungi the adsorption was of no relevance in relation to the PCP degradation (Table 3). In fact 95% of the PCP was degraded after 28 days of incubation by fungi *B. nivea* and *S. brumptii* are known to not produce extracellular enzymes as

lignin peroxidases or laccase which are highly efficient in degrading PCP in co-metabolism (McAllister et al. 1996; Field and Sierra-Alvarez, 2008). However, *S. brumptii* is a non-ligninolytic fungi that thanks to the phenoloxidase enzymes (Tanaka et al. 2000) can be useful in PCP degradation (Tomassini et al. 2001; Gadd 2001). *B. nivea* thanks to the pectinolytic enzymes, which are widely used in bioremediation strategy (Gadd 2001), was able to degrade 12.5 and 25 mg PCP L⁻¹ after 28 days in flask. Furthermore in the *Byssochlamys* genus there have been detected enzymatic activities involved in the degradation pathway of lignin and other wood constituents (Chu and Chang 1973; Furukawa et al. 1999). The ability of *B. nivea* and *S. brumptii* to remove PCP was excellent after 28 day of incubation. From the same soil from which we have isolated these fungi, Scelza et al. (2008) isolated a strain of *Byssochlamys fulva* that was able to remove 20% of 50 mg PCP L⁻¹ in only 8 days. Excellent results in PCP depletion were also obtained by Tomassini et al. (2001) with an isolate of *Rhizopus nigricans*. In fact the strain degraded 12.5 mg PCP L⁻¹ using phenoloxidase activity in only 8 days. Tortella et al. (2008) in N-limited liquid medium showed that *Anthracoerythium discolor*, *Lenzites betulina* and *Galerina patagonica* removed 25 mg PCP L⁻¹ in only 15 days thanks to manganese and lignin peroxidase production. Chiu et al. (1998) detected that the maximum total removal efficiency was obtained by *Ganoderma lucidum*, *Phanerochaete chrysosporium* and *Polyporus* sp. after 7 days of incubation in liquid batch system with 25 mg PCP L⁻¹ using laccase. These fungi were able to remove 75-78% of the PCP initial concentration. The majority of fungi implicated in PCP degradation are members of the white-rot Basidiomycetes and are capable of degrading lignin. Almost all studies on PCP degradation were conducted with *Phanerochaete chrysosporium*, *Phanerochaete sordida*, *Trametes versicolor* and *Trametes hirsuta* (McAllister et al. 1996). Few studies about PCP degradation by Ascomycota fungi are available in literature. This justifies the

importance to study fungi belonging to this fungal group that may tolerate and degrade high PCP concentrations.

4.5.3. Biological control of *P. cambivora* and *P. cinnamomi*

B. nivea and *S. brumptii* have demonstrated excellent capacity to reduce the growth of the pathogens in PDA and PDB dual culture methods. No significant difference was found between the growth and biomass inhibition of *P. cambivora* and *P. cinnamomi*. Several antagonists and their metabolites were found useful in *Phytophthora*'s control. A Flufluran derivate extract by *Asperigillus flavus* has completely inhibited the mycelium growth of *P. cinnamomi* after 7 days (Evidente et al. 2006). Antibiosis and mycoparasitism action by some fungi and bacteria, isolated by manure compost, were able to lyse *Phytophthora*'s mycelium with inhibition values between 40 – 70% (Aryantha and Guest 2006). B-glucosidase and phosphates activities of a *Trichoderma* sp. strain reduced the development of *P. cinnamomi* (Kelley and Kabana 1976). A methanolic compound obtained from the cyanobacterium *Nostoc* was tested with good results against a variety of pathogens of agricultural importance among which: *P. cambivora* and *P. cinnamomi* (Biondi et al. 2004). Antibiosis was detected only between fungi and *Phytophthorae* and, the main compound produced by our fungi able to limit the pathogens growth was patulin. Patulin is a mycotoxin active against a wide spectrum of microorganisms. Patulin produced by *Aspergillus clavatonaticus* exhibited inhibitory activity in vitro against several plant pathogenic fungi i.e., *Botrytis cinerea*, *Didymella bryoniae*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* (Zhang et al. 2008). This mycotoxin was able to stop the synthesis of rRNA, tRNA and mRNA in *Saccharomyces cerevisiae* (Sumbu et al. 1983) and to have antibacterial activities against *Escherichia coli* and *Micrococcus luteus* (Praveena and Padmini 2011).

In VOC experiments the oomycetes showed no significant decrease in radial growth when cultured with fungi. This happens because *B. nivea* and *S. brumptii* do not produce volatile compounds able to limit pathogens' growth. The VOCs are often used to control *Phytophthora* species. The endophytic fungus *Muscodor crispans* produced VOCs that have inhibitory effects against *P. cinnamomi*, *P. cambivora* and other oomycetes such as *Phytophthora palmivora*, *Pythium ultimum* (Mitchell et al. 2010). *Nodulisporium* sp., another endophytic fungus, produces VOCs inhibitors against a number of pathogens such as *Aspergillus fumigatus* and *Rhizoctonia solani*, *Phytophthora cinnamomi* and *Sclerotinia sclerotiorum* within 48 hr of exposure (Mends et al. 2012).

Ink disease, caused by the oomycete pathogens *P. cambivora* and *P. cinnamomi*, is probably the most important Chestnut disease . It is very destructive and causes flame shaped dark necroses on collar rot of adult trees, shrub and seedling. Biological control is an excellent alternative for sustainable agriculture to avoid problems associated with the use of pesticides. Many studies have been done so far ; although almost always conducted in the laboratory. In this regard, *B. nivea* and *S. brumptii* do not produce toxic compounds able to damage cell wall and membrane of tomato stem cell; as is shown with the ELA experiment (Table 3). In contrast, the incubation of tomato stem in *Aspergillus flavus* culture filtrates produces high phytotoxicity due probably to hydrophilic metabolites that remain in the aqueous phase (Evidente et al. 2009). In our experiment no significant difference in conductance was detected between the tomato stem incubated in distilled water and in *B. nivea* or *S. brumptii* culture filtrates. The next research step is to address in a field study the results obtained in this research.

4.6. Conclusions

Critical analysis reveals that there are relatively few reports on the use of microorganisms in the double role of bioremediation and biocontrol agent. This is the first paper about *B. nivea* and *S. brumptii*'s capacity to remove PCP and to control the pathogens agent of Ink disease caused by *Phytophthora* species. Furthermore, the excellent result showed by ELA assays on tomato stem indicate that our fungi do not cause significant damage to plants and that they can be used in field experiments. For this reason, our results indicate that *B. nivea* and *S. brumptii* have an interesting potential to be used in bioremediation and biocontrol strategy.

4.7. References

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Auricularia auricula

5. Bioaugmentation and biostimulation of an agricultural microcosm soil contaminated by pentachlorophenol

5.1. Abstract

Pentachlorophenol (PCP) has been used worldwide as a wood treatment agent and biocide. Its toxicity and extensive use has placed it among the most hazardous environmental pollutants. In this study we evaluated the response of an agricultural soil to contamination by PCP (25 mg Kg⁻¹), and to the adding of solid urban waste compost and two exogenous ascomycota fungal strains: *Byssoschlamys nivea* and *Scopulariopsis brumptii*. The experiments were conducted in microcosm soil incubated for 28 days at 25 °C. The depletion of PCP and the changes in biochemical soil properties (i.e. microbial biomass, soil respiration, dehydrogenase activity and fluorescein diacetate hydrolysis) were detected. Fresh soil showed an endogenous microbial activity indicated by soil respiration, microbial biomass and all the enzymatic activities tested. The addition of PCP severely depressed some of the tested biochemical properties suggesting an inhibitory effect on microbial activity. On the other hand, the compost limited the negative effect of PCP on the soil biochemical activity. After 28 days of incubation the compost and the fungal strains reduced to 95% the extractable PCP. The natural fresh soil showed a good efficiency in reducing extractable PCP (88%). Our results indicate that many processes (i.e. microbial degradation, biostimulation by compost and sorption to organic matter) were likely to occur in the contaminated soil when PCP was added and were involved in its depletion. Finally, we suggest that compost, *B. nivea* and *S. brumptii* can be successfully used to treat PCP polluted soils.

5.2. Introduction

Pentachlorophenol (PCP) is a toxic compound widely used as a wood treatment agent and general biocide. PCP is persistent in the environment and has been classified as a priority contaminant to be reclaimed in many countries. In fact uncontrolled PCP uses and releases has caused contamination of soil, water and ground water (McAllister et al., 1996). Although PCP is recalcitrant to biodegradation, numerous bacterial and fungal isolates have been reported to be able to degrade it (McAllister et al., 1996; Gadd 2001; Singh 2006; Field and Sierra-Alvarez 2008). The use of microorganisms for the depletion of PCP in contaminated soil and water has become an important alternative in bioremediation strategies.

Mycoremediation is a widely used process for this purpose and numerous fungi are capable to tolerate and remove PCP. White-rot and brown-rot fungi as *Phanerochaete chrysosporium*, *Antracophyllum discolor*, *Trametes versicolor*, *Ganoderma lucidum*, *Armillaria mellea*, *Gleophyllum striatum* have demonstrated to be able to degrade and mineralize PCP at very high initial concentrations and in a short time (McAllister et al., 1996; Field and Sierra-Alvarez 2008). At present only a few reports about PCP degradation by using fungi different from white-rot and brown-rot fungi are available in literature. This justifies the importance to study fungi belonging to other ecological fungal groups that may tolerate high PCP concentrations, grow faster and potentially increase degradation efficiency. Fungal strains belonging to the following taxonomic groups; *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp. and *Verticillium* spp., were tested in PCP tolerance and degradation in an extensive screening experiment (Seigle-Murandi et al. 1991;1992;1993). The ubiquitous fungus *Trichoderma harzianum* converted PCP in petachloroanisole (Rigot and Matsumura 2002). Tomasini et al. (2001) showed how an isolate of *Rhizopus nigricans* was resistant and able to degrade 12.5 mg of PCP L⁻¹ in 144 h. Furthermore concerning the *Rhizopus* genus, León-Santiestebán et al. (2011) discovered an isolate of *Rhizopus oryzae* that

was able to degrade 90% of 25 mg of PCP L⁻¹. In an extensive degradation experiment carried out by Carvalho et al. (2009) numerous species belonging to the *Penicillium* genus were able to degrade up to 50 mg of PCP L⁻¹.

Soil microbial degradation may be limited by several factors such as suboptimal nutrient levels, water content, temperature, pH level, organic matter and compost (McAllister et al. 1996). The use of compost material to biostimulate the microbial activity in PCP degradation was successfully applied in the depletion of contaminants in the soil (Semple et al. 2001; Jaspers et al. 2002; Miller et al. 2003; Jang et al. 2006; Scelza et al. 2008). Compost, being an excellent soil ameliorant for structure and composition with diverse microorganisms populations and a nutrient source for indigenous degraders, can be used in contaminated soils with excellent results (McAllister et al., 1996; Semple et al. 2001; Gadd 2001; Singh 2006; Field and Sierra-Alvarez 2008). The use of compost has enormous advantages over other bioremediation strategies; it has low operating costs and relatively high treatment efficiency. The compost used in these applications is commonly formed by manure, solid urban waste, yard wastes in which a fungi inoculation is often added to supplement the amount of nutrients and readily degradable organic contaminants in the soil (Barker and Bryson 2002).

In this study we evaluated, in a long-term experiment under controlled conditions, the efficiency of *Byssoschlamys nivea* (Westling, 1909) and *Scopulariopsis brumptii* (Salvanet-Duval, 1935) (Fungi: Ascomycota) (Fungi: Ascomycota) in depleting PCP in an agricultural soil microcosm, with no history of PCP contamination, with and without solid urban compost adding. In this experiment we also analyzed soil biochemical properties as microbial biomass, basal respiration, dehydrogenase activity and fluorescein diacetate hydrolysis.

5.3. Materials and Methods

5.3.1. Fungal strain and cultivation conditions

B. nivea and *S. brumptii* were stored into slant tubes containing potato dextrose agar (PDA) (5 g L⁻¹ potato; 20 g L⁻¹ dextrose; 15 g L⁻¹ agar) at 4°C in the laboratories of Forest Pathology of the Department of Agriculture (University of Naples Federico II, Italy) .

5.3.2. Chemicals

PCP (>99% purity) and HPLC solvent were obtained from Sigma Aldrich (Germany). All other chemical reagents were purchased by BD (United States of America).

5.3.3. Physical and chemical properties of soil and compost

Soil was collected (0 - 20 cm depth) from an agricultural lemon orchard land in southern Italy (Naples). This soil has no history of PCP contamination. The soil was sieved to 2 mm in field and sealed in a black box. In the laboratory the box was stored at 4 °C before analysis.

Physical and chemical soil analysis were carried out in triplicates on air-dried soil sample (Sparks, 1996). The texture was evaluated according to USDA Textural Soil Classification (Soil Survey Staff, 1975). potentiometric measurements of the H⁺ activity in a soil suspension in salt solution were carried out to determine the pH (Alef and Nannipieri 1995). Electrical conductivity (EC) was evaluated on the total concentration of dissolved salts (Violante 2000) while Cation Exchange Capacity (CEC) from the interaction between abarium chloride solution (exchanging solution) and triethanolamine (buffer solution) (Violante 2000). Water-holding capacity was determined analyzing the excess amount of water percolated through a known amount

of field-moist soil (Alef and Nannipieri 1995). Total nitrogen (TN) estimate was obtained with a sulphuric acid digestion and colorimetric analysis (Kjeldahl procedure) (Alef and Nannipieri 1995). Organic carbon (C) was performed by means of modified Walkley-Black method based on colorimetric determination after wet potassium dichromate digestion (Jackson 1958). Finally, Nitrate (NO_3^-) and nitrite nitrogen ($\text{NH}_3\text{-N}$) was detected using the procedure described in Wills et al. (1987).

The matured compost was obtained from solid urban waste and its composition is widely described in Scelza et al. (2007).

5.3.4. Soil biochemical analysis

Soil respiration (SR) was carried out after 1, 3, 7, 13, 18 and 28 days of incubation according to Piotrowska et al. (2006).

Microbial biomass-C (MB-C) was estimated with a fumigation-extraction methods that convert the extractable C to microbial C using standard factors (Vance et al. 1987).

Enzyme activities evaluated were: dehydrogenase (DH) and fluorescein diacetate (FDA). The first activity was an estimation of tetrazolium salt's (TTS) reduction to triphenyl formazan (TPF) (Trevors 1984). While the second was determined using the method based on the estimation of fluorescein produced in soil treated with fluorescein diacetate solution according to Schnüer and Rosswall 1982. MB-C, DH and FDA were analyzed after 1 and 28 days.

5.3.5. PCP depletion in microcosm soil experiment

PCP disappearance experiment in microcosm soil was performed in closed 1-l jars containing 100 g of fresh soil air-dried with 20% moisture content. The compost added was 7.14 g Kg^{-1} of soil corresponding to a field amount of $30 \text{ t compost ha}^{-1}$. The

samples with PCP were made by spiking the soil with 25 mg PCP Kg⁻¹ soil. To obtain a final concentration of 25 mg PCP Kg⁻¹ soil, we prepared a stock solution of PCP (5 g L⁻¹) in acetone. Approximately 10 g of soil was spiked with 7 ml of acetone and 0.5 ml of the PCP stock solution. In the jar, containing the two fungal strains, were inoculated 30 plugs (5 mm of diameter) of active mycelia from 7 days cultures days on PDA medium. When the fungi wereb simultaneously present 15 plugs were added for each isolate. The control was performed in the same experimental conditions without addition of inoculums, compost and PCP. All jars were incubated at 25 °C for 28 days in dark. For more details on microcosm soil preparation and spiked soil methods refer to Scelza et al. (2008). Experimental design is summarized in Table 8.

5.3.6. PCP extraction and quantification

PCP was extracted and analyzed according to protocol of Khodadoust et al. (1999). Briefly, 1 g of soil was extracted with 20 ml of water–ethanol 50+50 [v/v] and agitated of an horizontal shaker (190 rev min⁻¹) for 1 h. After which the supernatant was separated from the residual soil by centrifugation at 3000g for 15 min and then concentrated by evaporation under vacuum. Finally the concentrated samples were re-suspended in 2 ml of methyl alcohol (CH₄O) for high-performance liquid chromatography (HPLC) analysis. The residual amount of PCP was quantified in all the samples by HPLC using an Agilent Technologies R1100 instrument with a pump and a diode-array detector. A Phenomenex 250 x 4.6 mm C-18 column with 4 mm particle size and a Phenomenex C-18 (4.6 x 30 mm) guard column were used. Analysis was conducted using 68% of acetonitrile and 32% of buffered water (1% acetic acid) as mobile phase and the 1.0 ml min⁻¹ flow rate. Detection was carried out at 220 nm. The retention time for PCP was about 10 min. PCP extraction was evaluated to 1, 7,14 and 28 days.

Table 8. Agricultural microcosm soil experimental design. The symbols correspond: ● 30 plug (5 mm of diameter) of active mycelia from 7 day old cultures on PDA medium; □ 15 plug (5 mm of diameter) of active mycelia from 7 day old cultures on PDA medium for each fungus; * 7.14 g compost Kg⁻¹ soil; ▲ 25 mg PCP Kg⁻¹ soil.

Sample	Composition
S	Soil
SBN	Soil + <i>B. nivea</i> ●
SSB	Soil + <i>S. brumptii</i> ●
SBNSB	Soil + <i>B. nivea</i> □ + <i>S. brumptii</i> □
SC	Soil + Compost*
SBNC	Soil + <i>B. nivea</i> ● + Compost*
SSBC	Soil + <i>S. brumptii</i> ● + Compost*
SBNSBC	Soil + <i>B. nivea</i> □ + <i>S. brumptii</i> □ + Compost*
SP	Soil + PCP ▲
SBNP	Soil + <i>B. nivea</i> ● + PCP ▲
SSBP	Soil + <i>S. brumptii</i> ● + PCP ▲
SBNSBP	Soil + <i>B. nivea</i> □ + <i>S. brumptii</i> □ + PCP ▲
SCP	Soil + Compost* + PCP ▲
SBNCP	Soil + <i>B. nivea</i> ● + Compost* + PCP ▲
SSBCP	Soil + <i>S. brumptii</i> ● + Compost* + PCP ▲
SBNSBCP	Soil + <i>B. nivea</i> □ + <i>S. brumptii</i> □ + Compost* + PCP ▲

5.3.7. Statistical analysis

All statistics analysis were carried out using XLSTAT version 2013.1. Analyses of variance followed by Tukey's multiple comparison test at the 0.05 level was used to determine significant differences means. All experiments were performed in triplicates.

5.4. Results

5.4.1. Physical and chemical properties of soil

The texture analysis showed a soil with the following composition: sand (69%); silt (20%); and clay (11%). These values represent a loamy sand soil according to USDA Textural Soil Classification.

The principal characteristics of this volcanic soil can be summarized by highlighting the pH value at 7.53, 20% of moisture content, the organic matter of 34.25 g Kg⁻¹ of soil. Others physical-chemical properties of the soil are shown in Table 9.

Table 9. Main physical-chemical properties of the soil.

Analysis	Unit	Mean	SD
Electrical conductivity	μS cm-1	88.53	0.45
Moisture	%	20.02	1.10
pH		7.53	0.25
NNO3-	ppm	11.17	1.04
NNH3	ppm	3.43	0.93
Ctot	g Kg-1	19.87	1.53
Organic Matter	g Kg-1	34.25	2.64
N-kjeldhal	%	0.19	0.02
P	ppm	20.28	1.88
P2O5	ppm	46.66	4.34
K	ppm	165.33	7.57
K2O	ppm	200.05	9.16

5.4.2. Biochemical properties

SR without PCP was significantly affected by each factor considered up to 13 days. Then, the only significant difference was shown between samples with and without compost until the end of the experiment (Fig. 7a).

The increase of SR by PCP was evident after 1 day of incubation when the CO₂ produced in presence of PCP was 40 mg vs 45 mg of CO₂ for 100g⁻¹ of soil for the control (Fig. 7b). When both fungi or compost were added to the soil, the SR values were 2-fold higher than of the control after 13 days of incubation. After 18 days, the soil artificially contaminated with PCP had SR values twice as high than other samples. For all samples, the evolved CO₂ increased significantly up to 7 days and decreased after 13 days of incubation, with similar trend. At the end of the experiments all samples reached relatively similar values of evolved CO₂ (about 30 mg of CO₂ for 100g⁻¹ of soil).

The trend of MB-C was quite similar in samples with or without PCP. The MB-C values suffered a significant decrement from 1 day to 28 days of incubation for all samples in the controls. After 1 day of incubation the samples with compost showed high MB-C values (exceeding to 100 mg C 100g ss⁻¹) (Fig. 8a). The presence of PCP significantly decreased the MB-C values (Fig. 8b). At 28 day of incubation with PCP the MB-C showed the lowest values compared to the other samples (> 30 mg C 100g ss⁻¹).

Some enzymatic activities, involved in the cycle of the main biological nutrients, were evaluated. FDA and DH activity had an exactly opposite trend. Moreover, no significant difference in enzymatic activities was found between soil incubated with only *B. nivea* or *S. brumptii*. FDA values without PCP after 28 days of incubation were twice higher than the samples analyzed after one day (Fig. 9a). Even in this case the

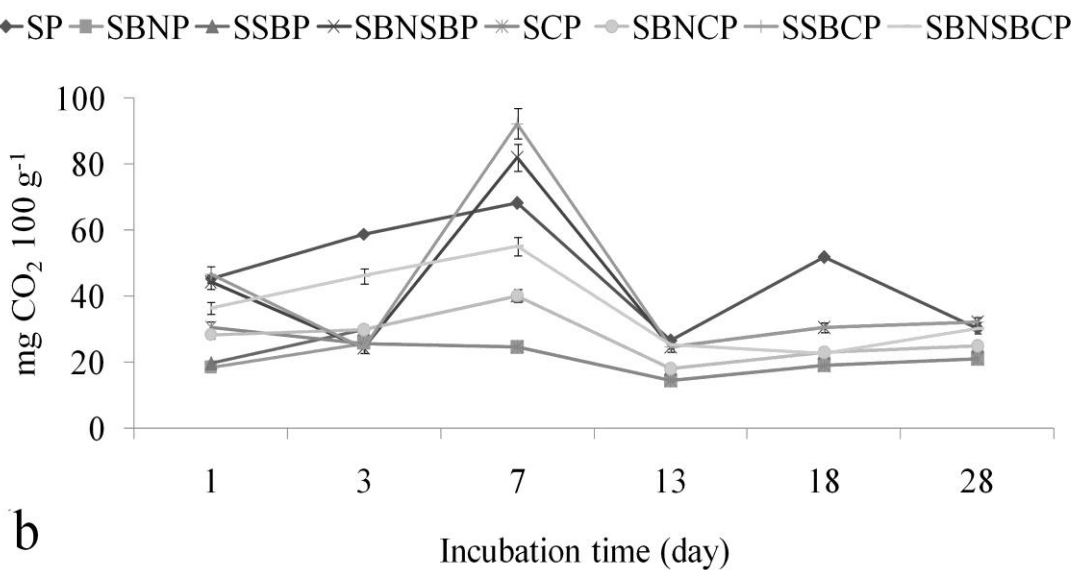
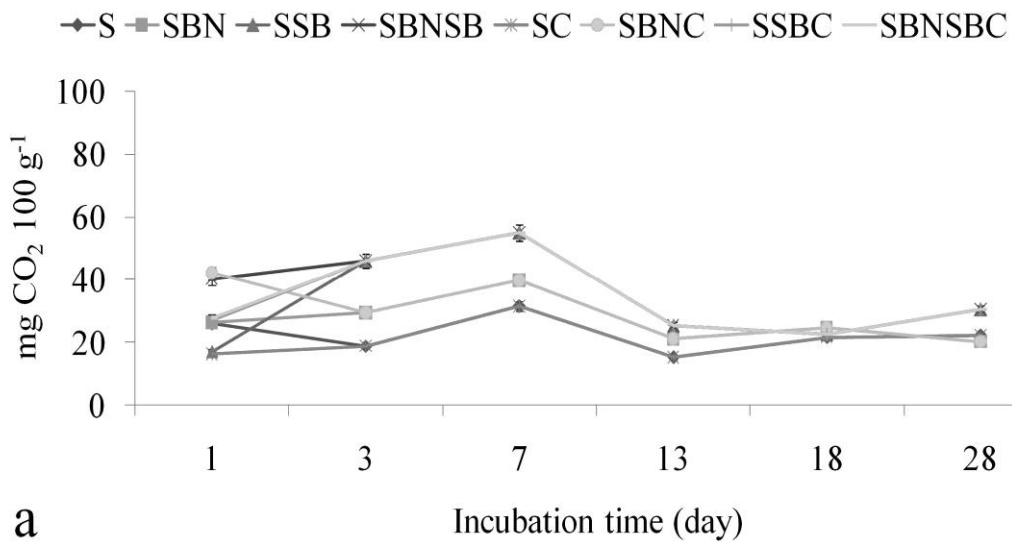


Fig. 7 Soil respiration of: a) control (without PCP);b) PCP contaminated sample (25 mg Kg⁻¹).

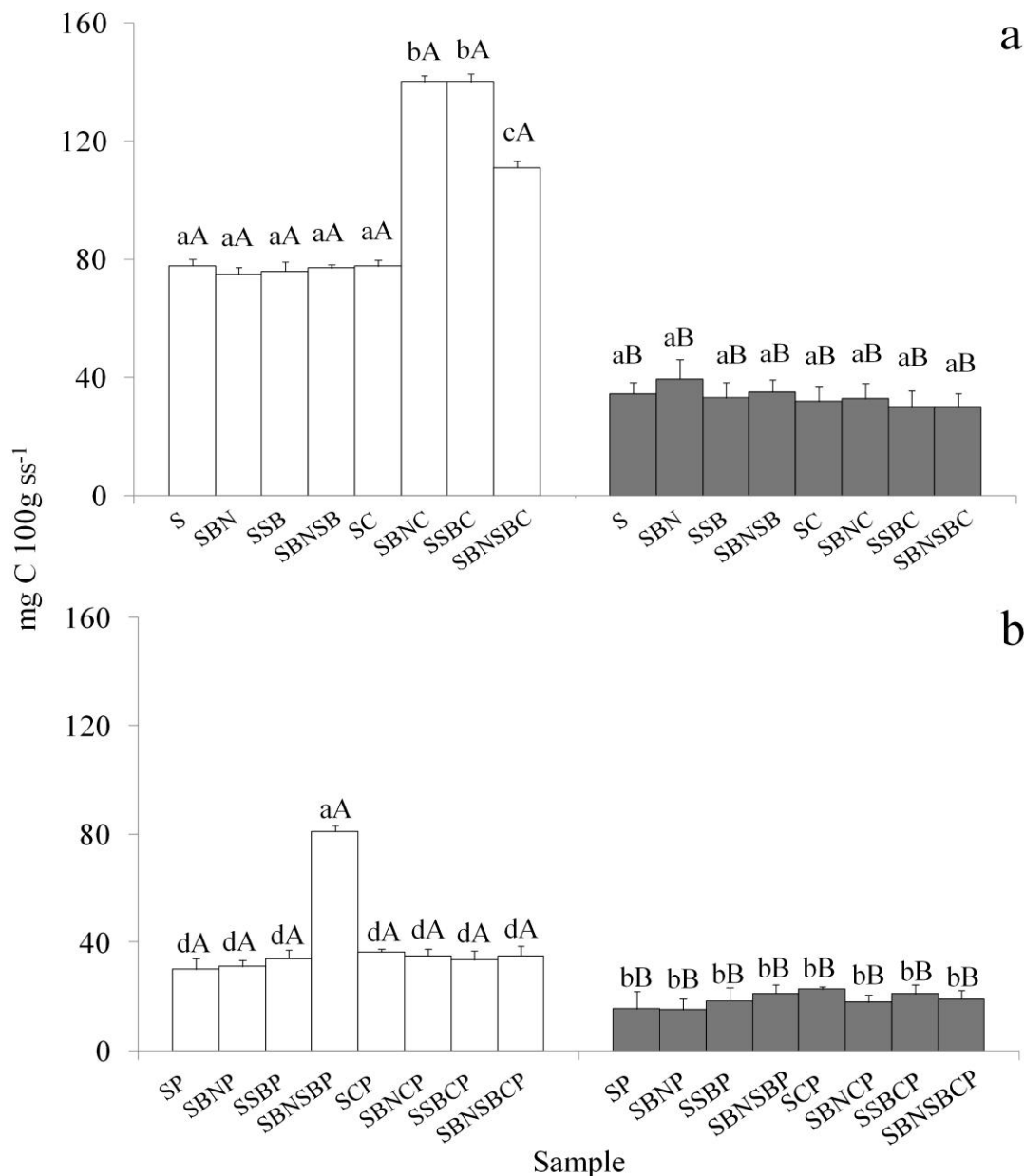


Fig.8 Microbial biomass of: a) control (without PCP); b) PCP contaminated sample (25 mg Kg⁻¹). Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

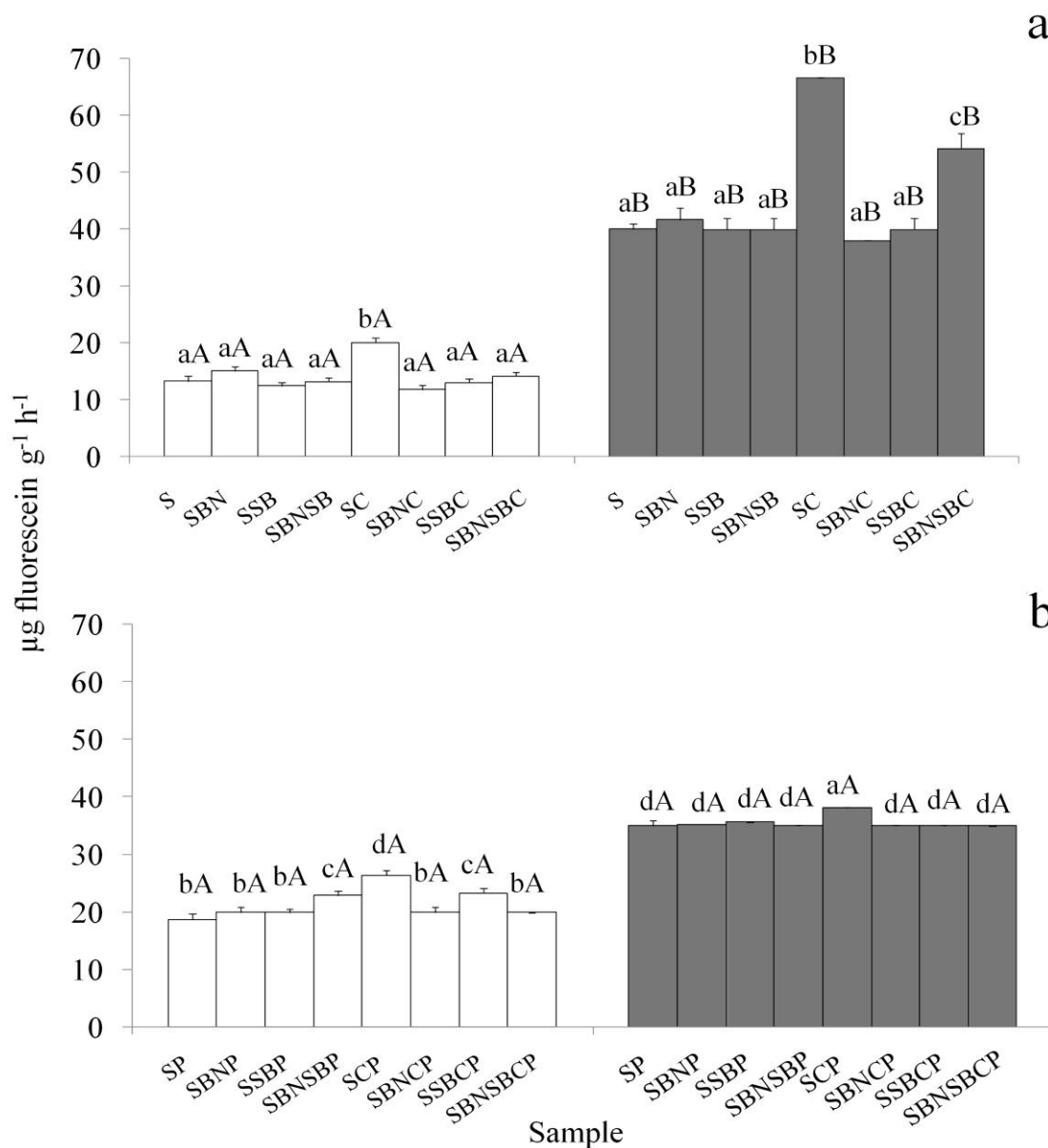


Fig.9 Fluorescein diacetate hydrolysis of: a) control (without PCP); b) PCP contaminated sample (25 mg Kg⁻¹). Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

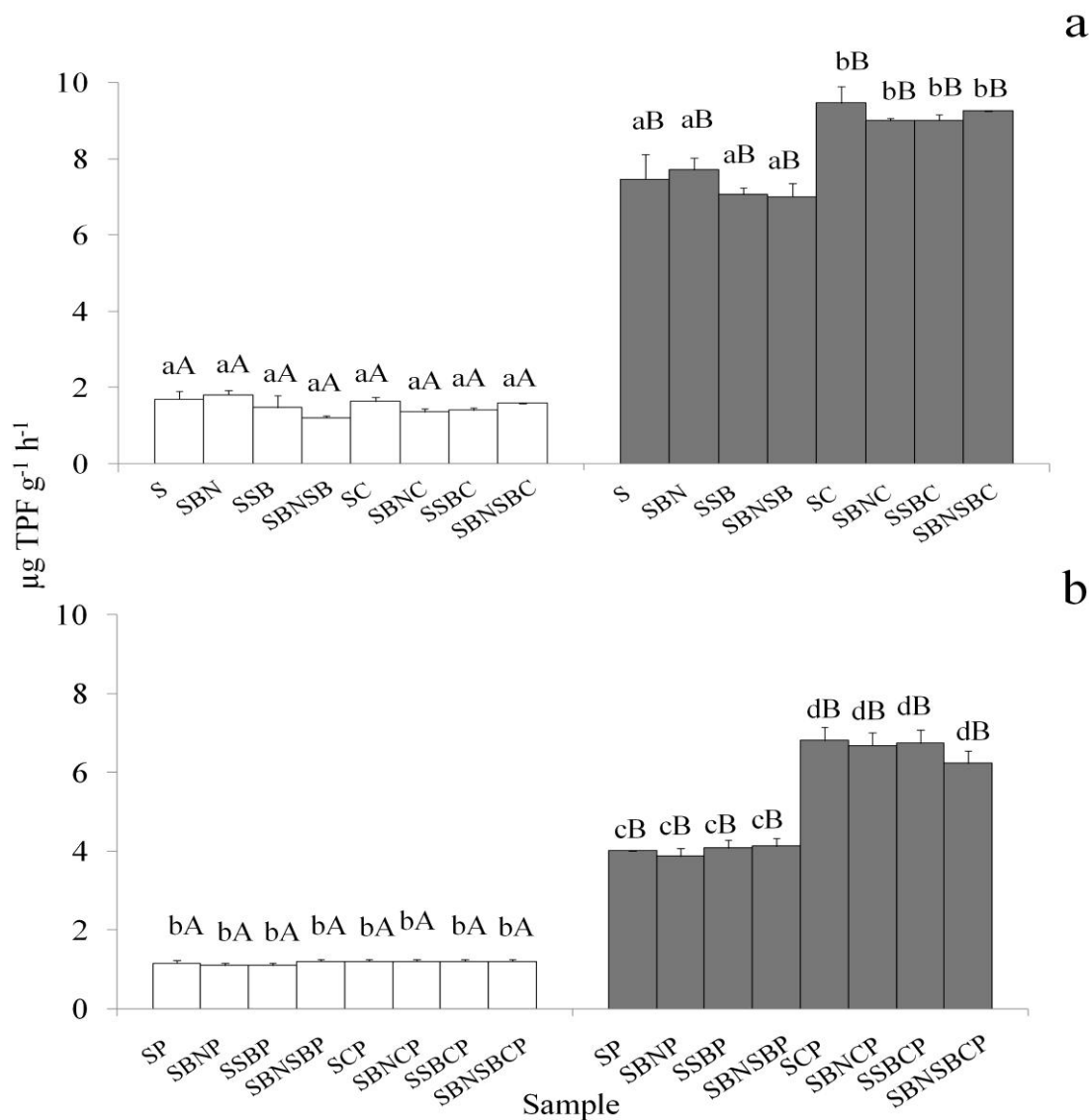


Fig.10 Dehydrogenase activity of: a) control (without PCP); b) PCP contaminated sample (25 mg Kg⁻¹). Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bars represent measurements carried out after: 24 h (white bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

samples with compost had the highest values of FDA ($> 60 \mu\text{g fluorescein g}^{-1} \text{ h}^{-1}$). When to the samples PCP was added, the FDA trend was similar to the samples without the contaminant (Fig. 9b). Furthermore, FDA values in a PCP contaminated soil were significantly higher than the control after 1 day of incubation, while, on the other hand, after 28 days of incubation, the FDA values were significantly lower than control. DH activity is a key enzyme in the C cycle and their values were significantly different in the sample with or without PCP and at different incubation times. DH values were higher after 28 day of incubation than after one day for all treatments. Moreover, when to the samples compost was added, the DH values were the highest of the whole experiment ($> 7 \mu\text{g TFA g}^{-1} \text{ h}^{-1}$) (Fig. 10a and 10b). In all cases when to the sample PCP was added, the DH values significantly decreased versus control without contaminant (Fig. 10b).

5.4.3. PCP depletion

Disappearance of PCP in soil, soil + compost, soil + compost + fungi and soil + fungi showed different trends. The extracted PCP amounts were significantly affected by soil treatment and incubation time. no significant difference was found in PCP removal between soil incubated with only *B. nivea* or *S. brumptii* (Fig. 5a and 5b). After 24 h of incubation a 40% reduction of extractable PCP was measured in all samples although samples composted and inoculated with both fungi showed a significant 45% reduction. For all sample, at 7 and 14 days, the PCP extracted was about 70% less than initial PCP concentration (25 mg Kg^{-1}). Even in this case the least amount the PCP recovered was for the soil with compost and both fungi. After 28 days of incubation almost a 95% of significant reduction of the extractable PCP was detected in soil sample with compost and both fungi vs. the natural soil sample (88% reduction of extractable PCP) (Fig. 11a and 11b).

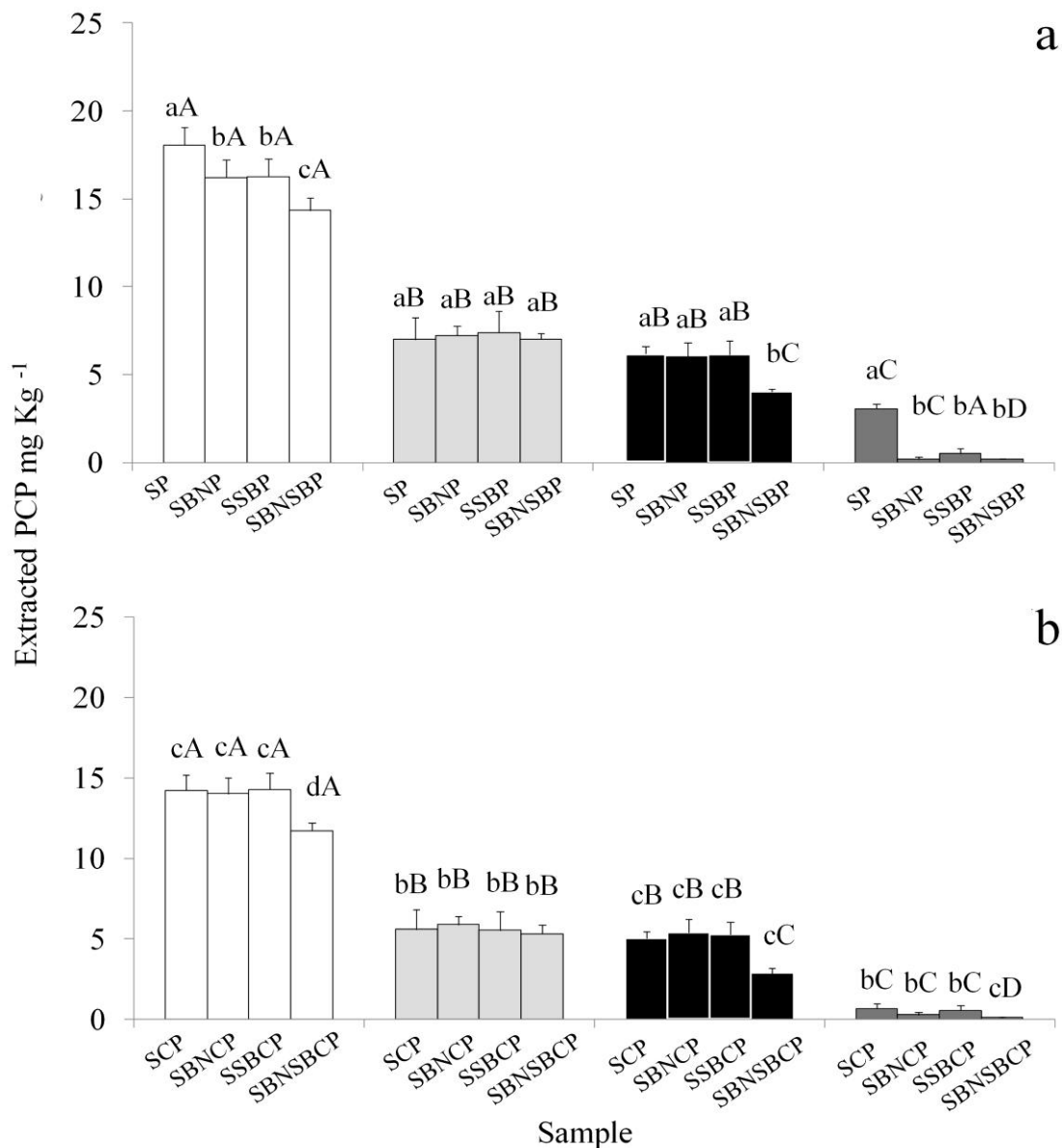


Fig. 11 Extracted PCP at different incubation times. Different small letters refer to significant differences ($P < 0.05$) among microbial biomass values of the different samples at the same incubation time. Different capital letters refer to significant differences ($P < 0.05$) among microbial biomass values of the same sample at different incubation times. Different color bar represent measurements carried out after: 24 h (white bar); 7 days (grey light bar); 14 days (black bar); 28 days of incubation (grey bar). Each value is the mean of three replicates.

5.5. Discussion

When Adding PCP to a natural fresh soil with no history of PCP contamination, several biochemical processes may occur in response to the contaminant. Different response were obtained if to the fresh soil compost and allochthonous fungi such as *B. nivea* and *S. brumptii* were added. The principal process that may occur in a soil when contaminated with PCP are: a) modification in biochemical soil activity; b) sequestration of PCP in soil matrix; c) degradation of PCP by bioaugmentation and biostimulation.

The investigated agricultural soil contaminated with 25 mg PCP Kg⁻¹ soil showed an intense microbial activity; as indicated from some biochemical properties observed during the incubation time. The PCP increased the SR in the soil already after 1 day and 1 week of incubation according to Scelza et al. (2008). In PCP contaminated soil, high SR activity indicates greater presence of fungi over bacteria, with the possibility of PCP degradation, resulting with the formation of its metabolites. The impact of low concentrations (25 – 50 mg/kg soil) of PCP seems to not negatively affect SR in soil at least in the initial phase of experiment. Zelles et al. (1989) detected that PCP concentrations of 2 and 20 mg Kg⁻¹ increased the SR for 90 days and at 200 mg PCP Kg⁻¹ there was an irreversible inhibition of SR. Instead, high concentration of contaminants (>100 mg PCP Kg⁻¹ soil) drastically reduced microbial respiration rates (Zelles et al. 1986). SR reduction of 20% in PCP contaminated soil applying electrokinetics studies was also detected by Lear et al. (2007) as consequence of the significant decline in microbial counts and biomass. Salminen et al. (1995) showed how SR decreased at the highest PCP concentration (500 mg PCP Kg⁻¹ soil) compared to the uncontaminated soil and soil with lower PCP concentration (50 mg PCP Kg⁻¹ soil). PCP is a wide spectrum biocide that has a negative impact on microorganisms diversity and soil activity. When PCP was added to fresh soil the MB-C decreased of 50% during all incubation times. Scelza et al. (2008) showed that the presence of PCP

severely and significantly depressed MB-C in soil contaminated by 50 mg PCP Kg⁻¹ soil. Many microcosms soil experiments detected a significant decreased in MB-C also when > 50 mg PCP Kg⁻¹ soil of PCP was added to the soil (Zelles et al. 1986; Salminen et al. 1995; Chaundri et al. 2000). Drastic MB-C decrement (89% less than control) with increasing soil PCP concentration was also showed by Megharaj et al. (1998). Soil enzymatic activities such as FDA and DH had an opposite trend. Exactly as Cea et al. (2010), FDA showed a good response to PCP in terms of total microbial activity especially during the early days of incubation. Similar results have been obtained also in other studies with indigenous microflora. Kähkönen et al (2007) found that PCP in a contaminated area with aged chlorophenols do not influence FDA. On the other hand DH activities was negatively influenced by PCP after 1 and 28 days of incubation with a 50% reduction vs the control. Same results were recorded with PCP values higher than our experiment. In fact with 50 and 250 mg PCP Kg⁻¹ soil, Scelza et al. (2008) and McGrath and Singleton (200), respectively, found that soil DH activity dramatically decreased and did not recover throughout the experiment (> 6 weeks). In contrast, Hechmi et al. (2014) observed that at low concentrations of PCP contamination the DH activity can slightly increase. Although in unplanted soils co-contaminated by Cd and PCP DH activity was significantly lower (64%) than the control value. Dh activity tended to decline with increasing PCP and Cd concentration in soil. The low values of all biochemical activities, indicative of microbial growth and often used as an index of PCP metabolite toxicity, indicate that PCP exerted a depressing effect on soil microbial activity although the extracted PCP decreased in time.

The fate of PCP can be influence by physical, chemical, and biological characteristics of the soil as well as the chemical properties of the contaminant. PCP Adsorption in soil is controlled largely

by its degree of substitution and the resultant hydrophobicity. At low pH values, where PCP exist mainly in a neutral form, the soil adsorbs much more PCP compared to the adsorption at higher pH, where PCP is present as an anionic form (Cea et al. 2007). At pH soil values (7 – 8), like our case-study, PCP is adsorbed to a less extent to the soil, although it can form irreversible bonds with soil even at high pH values (Abramovitch and Capracotta 2003; Cea et al. 2007). In general, as the pH comes closer to the pK_a , in this case 4.75, the adsorption increases due to the hydrophobicity of their neutral form (Cea et al. 2007).

Organic matter in soil is the most important sorbent for the phenolic compounds; in fact its presence in soil matter may enhance PCP sorption (Cea et al. 2007; Scelza et al. 2008). In our experiments, the soil has an organic matter value very high, about 34.25 g Kg^{-1} (Tab. 2) and this explains the drastic reduction (40%) of extractable PCP after only 24 h and a whole week. By increasing the organic matter in soil, adding compost or DOM, the sorption of PCP to the soil matrix is evidently favored (Jaspers et al. 2002; Miller et al. 2003; Scelza et al. 2008). Composts provides several microorganism populations and nutrients for indigenous degraders when applied to contaminated soils. Composting strategies have been successfully applied to PCP-contaminated soil, especially because the compost accelerates the PCP removal (Semple et al. 2001). With this aim, compost made with wood chips, sawdust, straw, farmyard manure and urban solid waste has provided excellent results to deplete PCP from contaminated soil (Semple et al. 2001; Scelza et al. 2008). In our study, the urban solid waste compost plays an important role to increase the reduction of extractable PCP and to limit the toxic effects of the contaminant on soil biochemical activities. After 28 day of incubation, the fresh soil treated only with urban solid waste compost removed almost 95% of PCP at an initial concentration of 25 mg Kg^{-1} soil. other excellent results were obtained always with a urban waste compost, which reduced the levels of PCP by 82% after one year (Field and Sierra-Alvarez 2008). In a similar study, contaminated soil

from a sawmill site was mixed with farm animal manure and composted. Complete depletion of PCP ($250 \text{ mg Kg}^{-1} \text{ dwt}$) rapidly occurred within 6 days (Jasper et al. 2002).

In relation to fungal strains used in PCP deletion, many studies have revealed that diverse members of Ascomycetes (*Paecilomyces* spp.) and Basidiomycetes (*Phanaerochaete* spp., *Trametes* spp. and *Bjerkandera* spp.) are efficient and fast degraders of PCP (Gadd 2001; Singh 2006; Field and Sierra-Alvarez 2008). When *B. nivea* and *S. brumptii* were added to fresh soil microcosm, PCP was reduced to 90 and 92%, respectively. Higher depletion of PCP occurred when fungal strains were simultaneously used. In fact the PCP reduction was 98%. *B. nivea* and *S. brumptii* do not produce extracellular enzymes as lignin peroxidases or laccase. It is widely known that these two enzymes are highly able to degrade PCP in co-metabolism (McAllister et al. 1996; Field and Sierra-Alvarez, 2008). However, *S. brumptii* is a non-ligninolytic fungi that thanks to its phenoloxidase enzymes (Tanaka et al. 2000) it can degrade PCP (Gadd 2001). Instead, *B. nivea* can deplete PCP thanks to its pectinolytic enzymes, which are widely used in bioremediation strategies (Gadd 2001). Furthermore, in the *Byssoschlamys* genus enzymatic activities involved in the degradation pathway of lignin and other wood constituent have been detected (Furukawa et al. 1999). Fresh soil (S), without compost or fungi, was already able to reduce 88% extractable PCP in 28 days of incubation. Although no attempts were made to detect the presence of PCP degradation products in the investigated system, the presence of its metabolites may be hypothesized according to Singh (2006), Field and Sierra-Alvarez (2008) and Scelza et al. (2008).

5.6. Conclusions

Our results showed that PCP produces a considerable reduction of the level of some biochemical properties i.e. thus suggesting a depressing effect on the soil microflora.

On the other hand, adding solid urban waste compost and fungal strains such as *B. nivea* and *S. brumptii*, it can possible to obtain a PCP reduction and an increase of the soil biochemical activities. Contrary to other studies where well known *white rot* or *brown rot* fungi are used to deplete PCP i.e. *Trametes versicolor*, *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, in this work, we have used ascomycota fungi novel in bioremediation studies, and indigenous microorganisms. The potential of certain fungi in field experiments can be enhanced by a number of factors. This includes an increase in the inoculum biomass/soil, or supplementing the soil culture system with compost. The compost and fungi were used in experiments controlled in laboratory conditions, further analysis may be helpful to obtain other result useful for field experiments close to natural situations.

5.7. Refernces

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Plerotus ostreatus

6. Concluding remarks

The aim of the present study, which is part of a wider bio-remediation project, was a long-term evaluation of the remediation ability of naturally selected fungi vs PCP, a biocide-chlorinated compound very toxic and dangerous for all components of the environment. In the first experiments were evaluated the PCP sensitivity, adsorption and degradation capacity of the two Ascomycota fungal strains selected, identified as *B. nivea* and *S. brumptii*. The second part of this project highlighted the existence of two strategies, biostimulation and bioaugmentation, for potential applications to bioremediation of PCP contaminated soils. In particular, biostimulation was explored by using a matured compost from solid urban wastes. Instead, *B. nivea* and *S. brumptii* were used in bioaugmentation experiments. The effectiveness of two bioremediation approaches was also evaluated against a PCP artificially contaminated soil.

The results “*in vitro*” demonstrated that *B. nivea* and *S. brumptii* were able to tolerate and remove PCP in liquid medium and in soil. Although *B. nivea* and *S. brumptii* do not produce extracellular enzymes as lignin peroxidases or laccase, that represent important tools to degrade the PCP in co-metabolism. These two fungal strain have other enzymes useful in PCP degradation i.e. phenoloxidase and pectinolytic enzymes.

Soil showed an intrinsic capability for PCP degradation, thus confirming that natural attenuation processes actually occurred. The presence of a high content of soil organic carbon, or the addition of organic matter to the soil as compost increase PCP depletion and mitigate the adverse effects that the contaminant may have on the biochemical soil processes. On the other hand, the addition of *B. nivea* and *S. brumptii* strongly stimulated and enhanced the attenuation process towards PCP detoxification in the freshly contaminated soil. Furthermore, several soil properties showed differentiated responses to the presence of PCP, compost, and/or exogenous fungi. In fact, when PCP

was added to the soil, the levels of some biochemical properties diminished over time, suggesting the occurrence of a depressing effect on the soil microflora which failed to recover from its initial toxic response to PCP. Conversely, compost and fungi, possibly contributing to PCP degradation and subsequent production of its metabolites, generally regarded as more toxic than the parent compound, developed in the PCP-contaminated soils. The temporary and permanent changes observed in several of the tested biochemical properties, in response to the presence of PCP, provide further support to the paradigm that soil biological investigations (such as those on soil respiration, biomass and enzyme activities) can give useful information on the status of soil quality.

In this thesis was also evaluated the adsorption capacity of the Chilean white rot fungus *Anthracophyllum discolor* in a liquid medium subjected to different pH values and initial PCP concentrations (5 and 10 mg Kg⁻¹). The experiment was carried out with live pellets of *A. discolor* pellets. The adsorption capacity of Chilean fungus was pH-dependent and was higher at a pH range of 5 – 5.5, while for other pH values it decreased significantly by < 60% at 10 mg PCP L⁻¹ in the pH range of 5.71 – 6.41. The FTIR analysis highlighted that amides, alkanes, carboxylates, carboxyl and hydroxyl groups can be important functional groups for the fungal adsorption of PCP. As previously shown for dead *A. discolor* pellets, live ones may be considered a good bio absorbent for liquid solutions contaminated by PCP.

Fungi and bacteria have been used by humans in many applications and play a role as natural environmental remediators. Microorganisms treatment of waste in nature has been known for centuries but most of our knowledge of the interaction between microorganisms and waste is based on studies performed in the laboratory. For the development of commercially viable bioremedial processes it is essential to link different disciplines such as microbial ecology, biochemistry, physiology and soil

science. During the last decade, fungi and bacteria have been used in the treatment of a wide variety of hazardous and toxic compounds in soil and sediments. Bioremediation has become an environmentally sustainable, attractive and promising alternative to traditional techniques for the remediation of persistent organic pollutants POPs contaminated sites. The key to successful bioremediation will be the implementation of the scientific and engineering work needed to provide sound basis for a correct and effective application to different research areas and real cases of environmental pollution.